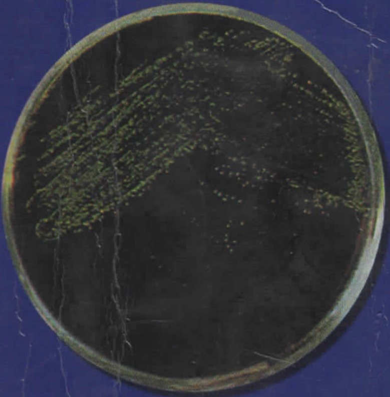
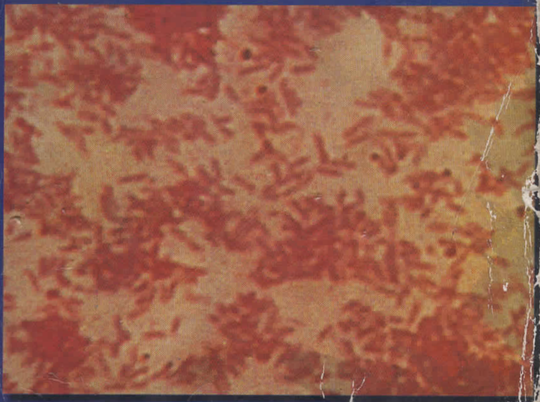
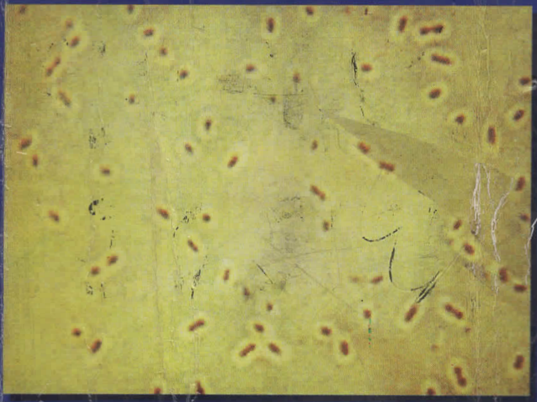
PRACTICAL MICROBIOLOGY



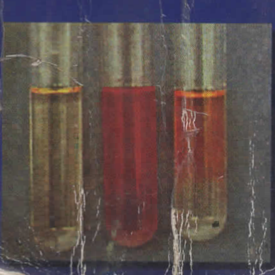


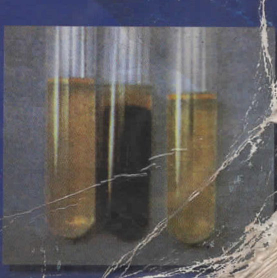


By

Pradeep Kumar Shah
Puspa Raj Dahal
Jyoti Amatya

2003





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[redacted]

PRACTICAL MICROBIOLOGY

By

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2003

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Preface

Microbiology is an important discipline which has contributed to the development of other subjects like Bio-chemistry, Molecular Biology, Biotechnology, Genetics and Genetic Engineering. In daily life man is in constant contact with the world of microbes, and various phenomena observed in the laboratory are merely examples of the complex interrelationships that play vital roles in human existence. Understanding of the interrelationships and their roles is the ultimate goal of the study of microbiology. Currently Microbiology is taught at under graduate and post graduate lavels in colleges and universities.

During our association in the teaching of microbiology and medical technology to undergraduate students we feel the need of an introductory book on the subject which can provide the requisite information in a simple style. The available texts written by foreign authors are generally voluminous, expensive and are meant for students who are already exposed to some microbiology source. Moreover these books are not adequately available in libraries. We believe that without some personal teaching/learning material, it is difficult to do justice to the subject. These considerations have prompted us to bring out this book with the hope that this book will facilitate the students and teachers to perform experiments in an easy way.

The authors are highly grateful to Prof. Dr. Sheetal Raj Basnyat, Former Head and Founder, Central Department of Microbiology, Tribhuvan University for going through the minuscript and for giving valuable suggestions in course of preparation of the book.

We are also grateful to Mr. Ashok Koirala and Amar B. Gurung, Asstt. Deans, Institute of Science and Technology, Tribhuvan University for graciously writing foreword of the book.

Any comments and suggestions for the betterment of this book will be highly appreciated.

P.K. Shah
P.R. Dahal
J. Amatya

Dedication

To our students and we hope this book helps to enrich their knowledge.

Foreword

This book entitled **PRACTICAL MICROBIOLOGY** written by teachers, *P.K. Shah, P.R. Dahal* and *J. Amatya* includes the practical being taught in B.Sc. level Microbiology course in the campuses of this country. Authors have made appreciating and genuine efforts to explain the theoretical aspects of principles involved in each practical, as well as to provide interpretation of facts involved in each practical in simple and understandable way.

As microbiology is coming up as one of the popular subject among the students in Nepal, it is hoped that this would be of use as a suitable book to cater the need of the microbiology students as well as for the basic researchers in the concerned field.

SRBasnyat

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FOREWORD

We are pleased to see the practical book entitled PRACTICAL MICROBIOLOGY for Microbiology students authored by our campus teachers P.K. Shah, P.R. Dahal, and J. Amatya.

We are confident that the book will minimize the shortage of appropriate books and prove beneficial to the students and teachers of Microbiology in enhancing their practical knowledge in Microbiology.

We wish for the fulfillment of its objective.

AMKoirala.
Ashok Koirala
Asstt. Dean

Amar B. Gurung
Asstt. Dean

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Section - I

Laboratory Safety: General Rules and Regulations

A rewarding laboratory experience demands strict adherence to prescribed rules for personal and environmental safety. The former reflects concern for your personal safety in terms of avoiding laboratory accidents. The latter requires that you maintain a scrupulously clean laboratory setting to prevent contamination of experimental procedures by microorganisms from exogenous sources.

Since you will be working with unseen living organisms it will be necessary that you develop a set of techniques that are new to you. Not only will these techniques determine the success or failure of your scientific probing, but they will also be essential to protect you and others around you against potentially harmful organisms.

Before you come to class, read over the exercise that is to be performed that day so that you understand what is to be done. The laboratory session will begin with a short discussion to brief you on the availability of materials and procedures. **Feel free to ask questions** when you do not understand the instructions or the principles that are involved.

Assume that all the organisms you work with are disease producing forms (Pathogens). Many of them will be harmless, but some are not. The following safeguards (rules) should be observed at all times.

1. Always wear a laboratory coat or apron before entering a laboratory for protecting clothes from contamination or accidental discoloration by staining solutions.

2. Before and after each laboratory work, clean your workbench with a disinfectant like lysol (1:500), phenol (1:100) or 90% ethanol.
3. Keep your laboratory bench clean of everything (e.g. books, purses, papers, etc.) except your laboratory equipment and notebook.
4. Never smoke, drink or eat in the laboratory. Make it a habit to keep your hands away from your face. Gummed labels should never be moistened with your tongue. Use tap water instead.
5. Never place pencils, labels or any other materials in your mouth.
6. If a live culture is spilled, cover the area with a disinfectant such as mercuric chloride for 15 minutes and then clean it.
7. In the event of a personal injury such as cut or burn, inform your instructor immediately as bacteria love open wounds.

8. Long hair should be tied back to minimize contamination of cultures and fire hazards.
9. Be careful of laboratory burners and turn them off when not in use.
10. All microbial cultures should be handled as being potential pathogenic.
11. The waste papers and contaminated glassware should be kept in receptacles provided.
12. Wash your hands with soap and water before leaving the laboratory.
13. Broth cultures must never be pipetted with mouth.
14. Aseptic technique must be rigorously observed at all times.
15. Always keep culture tubes in an upright position in a rack or basket.

16. Your must familiarize yourself in advance with the exercise to be performed.
17. Label all the plates, tubes, cultures properly before starting an experiment.
18. Materials such as stain and reagent bottles, Petri plates, pipettes must be returned to their original location after use.
19. Always clean microscope stage, eyepiece, lenses and objectives, before and after use. All lenses must be wiped off with the lens paper.
20. Never pipette by mouth any broth cultures or chemical reagents. Doing so is strictly prohibited. Pipetting is to be carried out with the aid of a mechanical pipetting device.
21. Speak quietly and avoid unnecessary movement around the laboratory to prevent distractions that may cause accidents.
22. Always perform exercises in sequence.
23. As you perform your exercises, record your data in ink and make sketches and labels in pencil.
24. Keep doors and windows closed during the laboratory session to prevent contamination from air currents.
25. Keep your lab notebook up-to-date.

1. Basic Requirements in Microbiology Laboratory

There are different types of equipments and materials used in Microbiology Laboratory. These equipments are used for various purposes such as for the sterilization, for the transformation of microorganisms and other materials, for the cultivation of microorganism and for the maintenance and storage of cultures and other materials. It is, therefore, essential to be familiar with the principles and procedures involved in handling these equipments.

Some of the common instruments used in microbiology laboratory are:

i) **Wire loops and needles**
ii) **Pipette**
iii) **Bunsen burner**
iv) **Waterbath**
v) **Hot air oven**
vi) **Autoclave**
vii) **Incubator**
viii) **Biological Safety Cabinet (Laminar Flow)**
ix) **Refrigerator.**

i) Wire loops and needles: Wire loops and needles are made from inert metals such as nichrome or platinum and are inserted into metal shafts that serve as handles. These are easily sterilized by incineration in the blue (hottest) portion of the Bunsen burner flame. Wire loops and needles are used for the aseptic transfer (sub-culture) of culture.

ii) Pipette: It is also used for sterile transfers. Pipettes are similar in function to Straws, that is, they draw up liquids. They are made up of glass or plastic drawn out to a tip at one end and with a mouth piece forming the other end. They are calibrated to deliver different volumes of liquid depending on requirements.

Pipettes may be sterilized in bulk inside canisters, or they may be wrapped individually in brown paper and sterilized in an autoclave or dry heat oven.

Precautions: Pipetting by mouth is not permissible; pipetting is to be performed with the aid of mechanical devices.

iii) Bunsen burner: The Bunsen burner, named after R.W. Bunsen, is a type of gas burner with which a very hot, practically nonluminous flame, the temperature of which reaches 1870°C at its hottest point, is obtained by allowing air to enter at the base and mix with the gas. The Bunsen burner or spirit lamp is used to sterilize inoculating loops/needles before and after they are inserted into cultures. It is also used for flaming the mouths of test tubes, media containing flasks and other glass apparatuses to avoid contamination by other microorganisms. Sterilization is done with dry heat. The materials to be sterilized are placed for a few seconds in a very hot (800-1800°C) flame that results in complete sterilization.

iv) Waterbath: It is an insulating metal box fitted with an electric heating mechanism and a thermostat which maintains the temperature at the desired level. There are racks for holding test tubes or flasks. The bath is filled with water until it rises almost to the top of the racks. Water baths are usually used for melting of media, testing enzymatic activities of various microorganisms, widal test etc.

The shaking waterbath is used for the cultivation of microorganisms in a broth medium. It is better than an incubator because it provides a rapid and uniform transfer of heat to the culture vessel and its agitation provides increased aeration, resulting in acceleration of growth and production of enzymes. The single disadvantage of this instrument is that it can be used only for cultivation of organisms in a broth medium.

v) Hot Air Oven: It is an instrument used in microbiology laboratory for sterilization process by dry-heat method. Hot air sterilization is recommended where it is either undesirable or unlikely that steam under regulated pressure (or autoclave) will make direct and complete contact with the materials to be sterilized, i.e., Heating by exposure to hot air is the accepted method for sterilizing load that cannot be really penetrated by steam and can tolerate the high temperatures (160-180°C) required.

Hot air oven is fitted with a fan to provide forced air circulation throughout the oven chamber, a temperature indicator, control thermostat and timer, and adequate wall insulation.

The sterilization hold time does not start until the chamber has reached the chosen sterilizing temperature and a further period has been allowed for all parts of the load to be raised to that temperature. For the sterilization, different time periods for different temperature is maintained. According to Medical Research Council, the commonly used time-temperature relations (table 1.1) are:

Table 1.1 : Time-temperature Relationship.

Temp. (°C)	Time (minutes)
140°C	120 min.
160°C	60 min.
170°C	40 min.
180°C	28 min.

Uses: It is used particularly for laboratory glasswares, such as test-tubes, petri dishes, flasks, pipettes, syringes and for metal instruments such as forceps, scalpels, and scissors. Besides these it is also used for oils, powders, and similar substances.

Precautions

1. All glassware to be sterilized should be perfectly dry before placing it in oven. Preliminary exposure in a drying oven at 100°C is recommended.

2. The powders, fats, oils and grease should be sterilized in sealed glass or metal containers in small amount not exceeding 10 gm in weight or 1 cm in depth.
3. The individual articles or packs of the load should be positioned to allow free circulation of the hot air between and around them.
4. It should not be attempt to open the chamber door until the chamber and load have cooled to below 60°C. Glassware is liable to crack if colder air is admitted suddenly while it is still very hot.
5. Articles like rubber goods, fabric or any inflammable or volatile substance should not be put inside.

vi) Autoclave: The laboratory apparatus designed to use steam under pressure for sterilizing heat stable materials is called an **autoclave**. Autoclaving provides moist heat at temperature higher than 100°C, the boiling point of water under normal atmospheric pressure. It does so by exposure of the load to the steam under increased pressure. This steam is biocidal. As it condenses on the cooler articles of the load, it releases both thermal energy (its latent heat) and moisture, which together denature microbial proteins. It has the advantage of rapid heating penetration and moisture in abundance, which facilitates the coagulation of proteins.

Although it is the temperature and not the pressure of the steam that confers the sterilizing effect, the tempemperature is determined by the pressure. The most efficient pressure-temperature combination to sterilize is 15 lb/in^2 which yields 121°C. It is possible to use higher pressure to reach higher temperature in emergencies and for sterilizing rubber and other materials that may be deteriorated with prolonged heat exposures. Time and temperature requirements for sterilization by steam under pressure in the autoclave are indicated in the table 1.2.

Table 1.2 : Time and Temperature Requirements.

Temp (°C)	Pressure above atmospheric pressure (lb/in^2)	Sterilization holding time (minutes)
115-118°C	10 lb/in^2	30 min.
121-124°C	15 lb/in^2	15 min.
134-138°C	30 lb/in^2	3 min.

A simple laboratory autoclave is very much similar to home pressure cooker in principle and consists of an upright metal tank heated below by electricity or gas and having a lid with a gasket enabling it to be fitted thermatically. The lid bears a manually operated tap for the discharge of air and steam, a pressure gauze and pressure stat (safety valve).

Uses: The autoclave is used to sterilize any thing that is not injured by steam and high temperature. This includes most types of solid and liquid media, solutions, rubber tubing, stoppers, discarded cultures and contaminated media prior to washing etc.

Precautions:

1. It is important to avoid over packing or haphazardly loading the chamber which prevents the steam from circulating freely around the content and impedes the full contact that is necessary.
2. Throughout the holding period of sterilization, the autoclave must contain only pure steam free from admixture with residual air. Before that period, therefore, air must be removed from the chamber.
3. After liquids have been sterilized (autoclaved), pressure should be returned to normal by exhausting the steam very slowly from the chamber.
4. All items should be packaged to prevent recontamination when removed from the autoclave.
5. Autoclaving is ineffective against organisms in materials impervious to steam. It can not be used for heat sensitive articles.

vii. Incubator: An incubator is controlled chamber where the required degree of temperature (optimum temperature) can be achieved to facilitate the microbial growth. Incubator may also control the content of atmospheric gases such as O_2 and CO_2 that may be required for the growth of certain microbes. During the incubation period ranging from a day to several weeks, the microbes multiply and produce some visible manifestation of growth.

Incubator is similar to hot air oven except that the former works at low temperature. For the routine culture in microbiology laboratory, small capacity incubator fitted with thermostat is generally used. It is supplied with shelves and has an inner glass door which closes on a gasket to reduce heat loss. The operating temperature generally ranges from 5°C to 80°C with a 0.5°C fluctuation at 38°C and ±2°C temperature variation. A thermometer has been fitted at the upper inner part of incubator and air is circulated in it by natural convention. The maintenance of temperature for different microbes are as follows:

For phychrophilic organisms, optimum temperature is 15-20°C.
For Mesophilic organisms, optimum temperature is 37°C.
For thermophilic organisms, optimum temperature is 45°C or above.
For human pathogens, the temperature is maintained at 37°C for 24 to 48 hours.

Precautions:

1. The incubator should always be positioned on a level surface.
2. The temperature should always be examined before incubating anything.
3. Incubator should be cleaned regularly.
4. Glass door should not be opened for a long time.

viii. Biological Safety Cabinet (Laminar Flow): A biological safety cabinet is a device that encloses a workspace in such a way as to prevent workers from exposure to infectious agents, i.e. using a safety cabinet, germ-free environment (air) is created at working area.

Air that has passed over the infectious material is sterilized, either by heat, uv light or most commonly by passage through a high efficiency particulate air (HEPA) filter that remove most particles larger than 0.3 μm in diameter. These cabinets are designated by class according to the degree of biological contaminant they afford. There are three kinds of safety cabinet – class I, II and III. Most hospital clinical microbiology laboratory technologists use class II cabinets for routine use. e.g. Inoculating, culture transferring etc.

Precautions

1. A safety cabinet should not be loaded with unnecessary equipments. Otherwise it will not carry out its job properly.

2. The operator should avoid bringing the hands and arm out of the cabinet while working.
3. Safety cabinets should be swabbed out with a suitable disinfectant after use and regularly decontaminated with formaldehyde.

ix. Refrigerator: A refrigerator is used for a wide variety of purposes such as maintenance and storage of stock cultures between subculture period and storage of sterile media to prevent dehydration. It is also used as depository for thermolebile solutions, antibiotics, serums and biochemical reagents.

Besides these instruments different types of other glassware such as test tubes and petri-plates are also used to cultivate microorganisms.

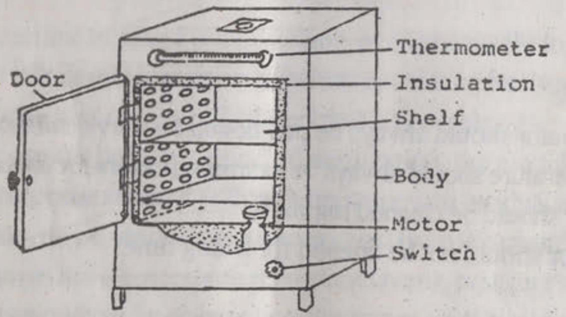
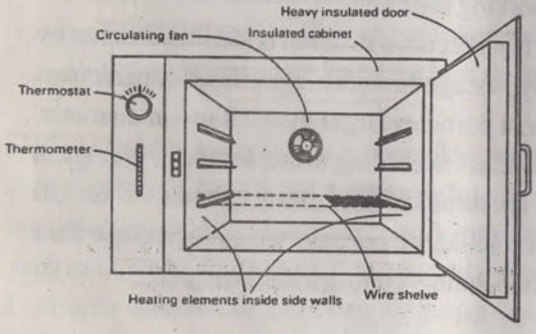


Fig. Incubator.



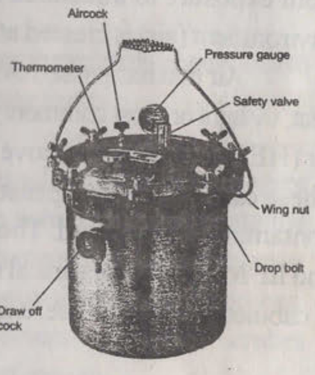


Fig. Hot Air oven.

Fig. Autoclave.

Section - II

2. The Microscope

In order to understand the indispensable role played by the microscope in the study of microorganisms, it is necessary to appreciate the intrinsic limitations of the eye as a magnifying instrument. The apparent size of an object viewed by the human eye is directly related to the angle that the object subtends at the eye; hence, if its distance from the eye is halved, its apparent size is doubled. However, the eye cannot focus on objects brought close to it than approximately 25 cm; that is, accordingly, the distance of maximal effective magnification. In order to be seen well, an object must subtend an angle at the eye of 1° or greater; and for a distance of 25 cm, this corresponds to a particle with a diameter of approximately 0.1 mm.

Most cells and hence the unicellular microorganisms are too small to be detected by the unaided human eye. In order to detect them, and to observe their form and structure, the use of microscope is therefore essential. The function of magnifying lens system of this instrument, interposed between the specimen and the eye, is to greatly increase the apparent angle subtended at the eye by objects within the microscopic field. In addition to this factor of magnification, two other factors, contrast and resolution, are of great importance.

It was Antony Van Leeuwenhoek, a Dutch, who for the first time in 1676, used simple microscope consisting of a biconcave lens enclosed in two metal plates to describe bacteria and protozoa. These simple microscopes magnified objects 200-300 times with which only the gross form of cells could be observed. The inventors of the compound microscopes, which consisted of a two lens system, is still in dispute but it is true that Robert Hooke had used a compound microscope in eighteenth century. A modern compound microscope contains three lens systems. The condenser interposed between the light source and the specimen collimates the light rays in the plane of the microscopic field. The objective produces a magnified image of microscopic field within the microscope; and the ocular further enlarges this image and enables it to be perceived by the eye.

Single lenses have two inherent optical defects. They fail to bring the whole microscopic field into simultaneous focus (Spherical aberration), and they produce coloured fringes around objects in the field (chromatic aberration). These defects can be largely eliminated by placing additional, correcting lenses adjacent to a primary magnifying lens. Consequently, both the ocular and objective lenses of a modern compound microscope are multiple ones, designed to minimize these aberrations. Correct adjustment of the condenser lens is of critical importance in providing a clear image. When a microscope is used at high magnifications, the condenser is usually positioned in order to provide Koehler illumination, in which specimen is illuminated by parallel rays of light.

There are two categories of microscopes, namely (1) light microscopes using optical lenses and light rays and (2) Electron microscopes using electron beam and electromagnetic lenses in vacuum. Light microscopes may work under the principle of (a) Bright field microscopy, (b) Dark field microscopy (c) Phase-contrast microscopy, or (d) Fluorescence microscopy. Likewise, electron microscopes may be (a) transmission or (b) Scanning ones.

The normal student microscope is a compound, light microscope working under the principle of bright field microscopy. Here, the microscopic field is brightly lighted up and the objects appear dark because of light absorption. Ordinarily, microorganisms do not absorb much light, but different dyes used to stain them lead to light absorption resulting in contrast and colour differentiation. The microscope normally used in microbiological laboratory may be monocular (one eye-viewing) or binocular (two eye-viewing).

The components of a microscope can be broadly categorized into (a) mechanical parts and (b) optic parts.

The metal structures of the microscope are the mechanical parts. The **base** or **the foot** of the microscope is the lowermost part, which supports the instrument. The U-shaped structure hinged to the foot at the inclination point is called the **arm or limb**. It bears a **stage** below and the **body tube** above. The object, which is to be studied, is placed on the stage. The stage also bears **slide clips** and field changing knobs. The body tube holds **lenses**. It can be moved up and down by rotating two focusing knobs. The bigger knob is called coarse focusing knob (**coarse adjustment**) and the smaller, the fine focusing knob (**fine adjustment**). These knobs help in focusing the specimen.

The optical parts lying below the stage are the accessories while those contained in the body tube are essentials (necessaries), which are directly involved in magnification. The lower part of the tube holds objective lenses of magnifying power 10X (20X), 40X (45X), and 100X, namely low power objective, high dry objective, and oil immersion objective respectively. These lenses are mounted on a revolving nosepiece which can be rotated to bring the desired lens in place. The upper end of the tube holds the eyepiece (**ocular lens**), which may be one of the 5X, 10X, or 15X magnifying lenses. These lenses are often replaceable. The important optical accessories of a microscope are **substage condenser** combined with **iris diaphragm** and a light source, either a double-faced **mirror** or a **light bulb**. The light source illuminates the object. The iris diaphragm is used to adjust the intensity of light and the condenser for focusing light to the object (by rotating the condenser knob near the inclination point).

Magnification means simply the increase in the size of image of an object. A compound microscope magnifies the image of an object twice. The objective forms a real and enlarged image which is further magnified by the eye piece into virtual image. Therefore, the total magnification is the product of the magnifying power of the objective and that of the eye piece.

Table 2.1: Magnification table

Objective	Eye piece		
	5X	10X	15X
10X	50X	100X	150X
40X	200X	400X	600X
100X	500X	1000X	1500X

*Generally, an eye piece having 10X is used, while the objectives are variable. Magnification can also be calculated as

$$\text{Magnification} = \frac{\text{Distance between nose piece and objective}}{\text{focal distance of objective}} \times \text{Magnification of eye piece}$$

Distance between nose piece and objective is usually 160 mm and focal distances of 10X, 40X and 100X objectives are usually 16mm, 4mm and 1.6 mm respectively. Therefore, when the eye piece used is of 10X,

For low power objective,

$$\text{Magnification} = \frac{160\text{ mm}}{16\text{ mm}} \times 10\text{ x} = 100\text{ x}$$

For high dry objective

$$\text{Magnification} = \frac{160\text{ mm}}{4\text{ mm}} \times 10\text{ x} = 400\text{ x}$$

and for oil immersion objective

$$\text{Magnification} = \frac{160\text{ mm}}{1.6\text{ mm}} \times 10\text{ x} = 1000\text{ x}$$

For low power and high dry objectives, the medium between lens and object is air but for oil immersion objective, medium is oil. Therefore, while using oil immersion objective, a drop of oil (cedar oil, paraffin oil or synthetic oil) should be applied on to the specimen. The oil used prevents light loss due to diffraction i.e. focuses more light to the specimen being observed at high magnification.

Maximum magnification is found in oil immersion objective. Field of view also changes with change of objective which is maximum with low power objective and minimum with oil immersion objective.

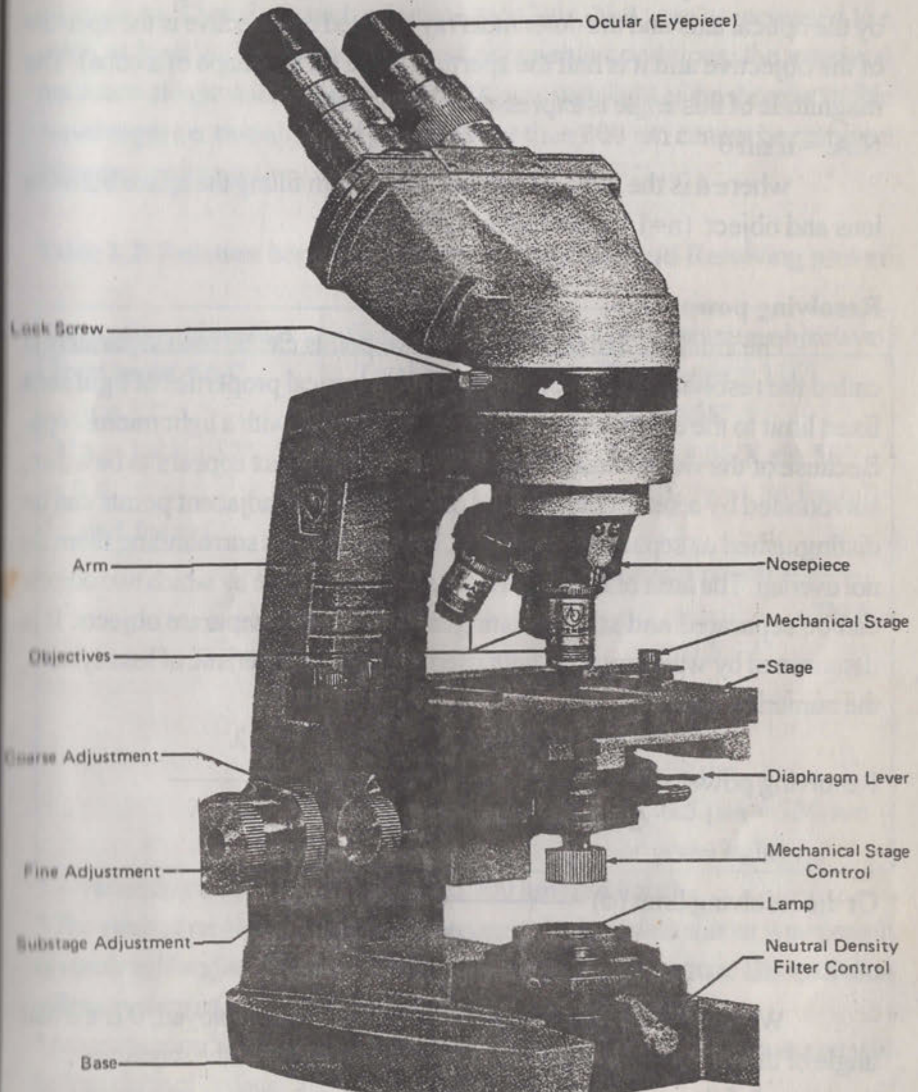

Ocular (Eyepiece)
Lock Screw
Arm
Nosepiece
Mechanical Stage
Objectives
Stage
Coarse Adjustment
Diaphragm Lever
Mechanical Stage Control
Fine Adjustment
Lamp
Substage Adjustment
Neutral Density Filter Control
Base

Fig. Parts of A Microscope.

Numerical Aperture

The numerical aperture (N.A.) can be defined as function of the diameter of the objective lens in relation to its focal length. The angle θ subtended by the optical axis and the outer most ray covered by objective is the aperture of the objective and it is half the aperture angle (in the shape of a cone). The magnitude of this angle is expressed as a sine value.

$$\text{N.A.} = n \sin \theta$$

where n is the refractive index of medium filling the space between lens and object. (n=1 for air and n=1.56 for oil)

Resolving power

The smallest distance at which two points can be seen separately is called the resolving power of the lens. The physical properties of light set a fixed limit to the effective magnification obtainable with a light microscope. Because of the wave nature of light, a very small object appears to be a disc, surrounded by a series of light and dark rings. Two adjacent points can be distinguished as separate or resolved, only if the rings surrounding them do not overlap. The limit of resolution is the smallest distance by which two objects can be separated and still be distinguishable as two separate objects. It is determined by wavelength of light used and the characteristic of lens system, the numerical aperture.

$$\text{Resolving power} = \frac{\text{Wave length of light } (\lambda)}{2 \times \text{Numerical aperture}} = \frac{\lambda}{2\ \text{N.A.}}$$

$$\text{Or the resolving limit } (d) = \frac{0.5\ \lambda}{n \sin \theta}$$

Where λ is the wavelength of the light source employed, θ is the half angle of the objective lens and **n** is the refractive index of the medium.

$$\text{or } d = \frac{0.5\ \lambda}{\text{N.A.}}$$

Upto certain limit, an increase in the numerical aperture of the objective lens increases resolving power. If the medium is air, a feasible diameter of the objective lens limits NA to approximately 0.65. The value of n is increased in oil medium. Therefore, with oil immersion lens, N.A. can be increased to a value as high as 1.4. Under the best obtainable conditions, the maximal resolution of light microscope reaches 200 nm, with light of the shortest visible wavelength i.e. two adjacent points closer than 200 nm cannot be resolved into separate images.

Table 2.2: Relation between Numerical Aperture and Resolving power.

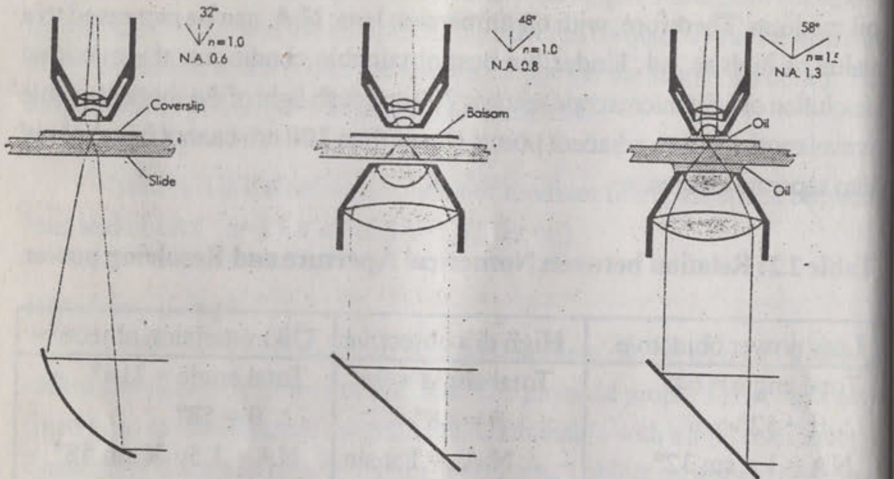
Low power objective	High dry objective	Oil immersion objective
Total angle = 64° $\therefore \theta = 32^\circ \therefore$ $\text{NA} = 1 \times \sin 32^\circ$ $= 0.6$ $[\therefore \eta = 1$ for air]	Total angle = 96° $\therefore \theta = 48^\circ$ $\therefore \text{N.A.} = 1 \times \sin 48^\circ = 0.8$	Total angle = 116° $\therefore \theta = 58^\circ$ $\text{NA} = 1.56 \text{ X} \sin 58^\circ$ $= 1.33 [\therefore \eta = 1.56$ for oil] $\text{Resolving power} = \frac{\lambda}{2\text{NA}}$ $= \frac{0.5}{2 \text{X} 1.33}$ $= 0.19\ \mu\text{m}$ $[\lambda = 0.5\ \mu\text{m} = 550$ nm for green light)

* λ varies from 400 nm (blue light) to 700 nm (red light)

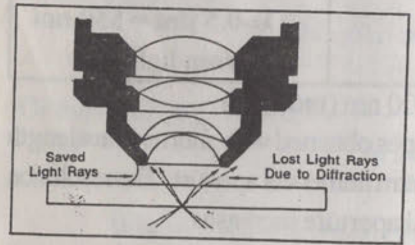
* The greatest resolution in light microscopes obtained with shorter wavelength of visible light and an objective with maximum numerical aperture. The resolution of lens system increases as the numerical aperture increases.

* Magnification beyond resolving power is of no value since larger image will be less distinct in detail and fuzzy in appearance.

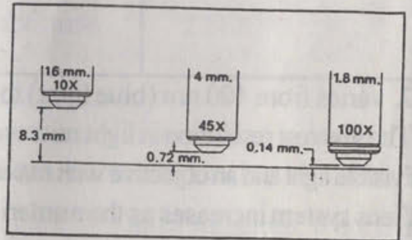
* The smallest details that can be seen by typical light microscope are those having dimensions of approximately. 0.2 μm (objects smaller than nearly 0.2 μm cannot be seen)



The relationship between angular aperture and resolution.



Immersion oil on the left side prevents light loss due to diffraction.



Working distance of objectives decreases with magnification increase.

Procedure for operating a microscope

1. Position the microscope firmly on the working table.
2. Clean the lenses with lens paper. If the lens has stains, oily substances, wipe it first with lens paper moistened with xylol and immediately wipe off xylol with lens paper dipped in 95% ethanol then wipe off lens with a dry lens paper.
3. Connect the bulb of microscope to power point. If there is no bulb, face the microscope to light source e.g. microscopic lamp. Adjust the mirror so that the flat side reflects light to the barrel of microscope.
4. Place the slide on the stage and secure it with stage clips for other means provided.
5. Bring low power objective in position by rotating the nose piece.
6. Look through eye piece and raise the condenser until there is maximum illumination (like a bright full moon).
7. Keeping both eyes open, bring the specimen into sharp focus by using coarse and fine adjustments.
8. Adjust iris diaphragm to produce optimum illumination.
9. Rotate nose piece to bring high dry objective in position without raising body tube. Get sharp focus by slight adjustment of fine focusing knob.
10. Swing the high power objective partially out of way and place a drop of immersion oil on the area of slide you are observing. Bring the oil immersion objective into position.
11. Again focus using fine focusing knob. If required, increase the light intensity.
12. After completion of your exercise, move the nose piece to bring low power objective into position, remove the slide, and clean the lenses.

Precautions

1. Always keep the microscope free of dust and moisture.
2. Keep it covered when not in use.
3. Do not touch the lenses with fingers. Fatty acids in perspiration may damage them.
4. Paper towelling or cloth should never be used on a lens surface. Always use lens paper or lint free 'optically safe' tissues.

5. Use proper side of mirror for proper illumination. (The concave side of mirror is used for natural light i.e. sunlight and the flat side for the artificial light e.g. lamp).
6. Do not remove the eyepiece from the instrument without covering the open end of the barrel with lens tissue.
7. Clean the lenses before and after carrying out an experiment.
8. If you are using a microscope in an inclined position, always return it to the original position after your work.
9. Carry only one microscope at a time grasping the microscope arm firmly with the right hand and the base with the left hand.
10. Do not tilt the microscope while using oil immersion objective. The stage should be kept in level to prevent the oil from flowing off the slide.
11. When using high dry or oil immersion objectives, never look down through the microscope while closing the distance between the objective and the slide. Observe this moment from the side of the microscope.’
12. Never use a slide without cover slip while examining an object under high power of the microscope, otherwise the chemicals will damage the objectives.
13. Observe the slide with both eyes open to have less strain on your eyes.
14. The immersion oil should be completely clear.
15. The low power objective is placed in position and body tube is lowered completely before returning the microscope to its cabinet.

Table 2.3 : Comparison of Various Types of Microscopes.

TYPE OF MICROSCOPE	MAXIMUM USEFUL MAGNIFICATION	RESOLUTION	DESCRIPTION
Bright-field	1,500 X	100-200 nm	Extensively used for the visualization of microorganims; usually necessary to stain specimens for viewing.
Dark-field	1,500X	100-200 nm	Used for viewing live microorganisms, particularly those with characteristic morphology; staining not required; specimen appears bright on a dark background.
Ultraviolet	2,500X	100 nm	Improved resolution over normal light microscope; largely replaced by electron microscopes.
Fluorescence	1,500X	100-200 nm	Uses fluorescent staining; useful in many diagnostic procedures for identifying microorganisms.
Phase contrast	1,500X	100-200 nm	Used to examine structures of living microorganisms; does not require staining
Nomarski differential interference	1,500 X	100-200 nm	Used to examine structures of microorganisms; produces sharp, multicoloured image with three-dimensional appearance.
Confocal scanning	1,500 X	100-200 nm	Used to examine structures of microorganisms and individual microorganisms within mixtures of various types of microorganism; uses fluorescence staining; produces blur-free image; used to produce three dimensional images.
TEM	500,000-1,000,000X	1-2 nm	Used to view ultrastructure of microorganisms, including viruses; much greater resolving power and useful magnification than can be achieved with light microscopy.
SEM	10,000-1,000,000X	1-10 nm	Used for showing detailed surface structures of microorganisms; produces a three-dimensional image.

3. Microscopic Measurement of Microorganisms

The measurement of microorganisms is known as micrometry. The dimensions of microorganisms are usually expressed in units smaller than millimeters. The micrometer (µm) is one thousandth (10^{-3}) of a millimeter, one millionth (10^{-6}) of a meter and 1/25,400 of an inch. Microorganisms such as virus are measured in nanometer and angstrom. A nanometer is 10^{-9} of a meter. An angstrom is one-tenth of a nanometer or one-ten-billionth (10^{-10}) of a meter. Determination of size of a microorganism is one of the properties useful for identification in the laboratory. Since microorganisms can be seen only under a microscope, their size can be measured by equipping the microscope with an ocular micrometer, which is then calibrated against a stage micrometer. An **ocular micrometer** consists of a circular disc of glass which has graduations engraved on one surface. It has 100 equally spaced divisions, marked 0-10. The distance between the graduations of an ocular micrometer does not have any standard value and varies depending on the objective used. This distance is found out by calibrating it with a known scale, the stage micrometer. A **stage micrometer**, which is also called an objective micrometer, has scribed lines on it that are exactly 0.01 mm (10 µm) apart.

Ocular micrometer after putting inside the eye piece is calibrated by superimposing the graduations of ocular micrometer over graduations of the stage micrometer, which is accomplished by rotating the ocular lens. And the number of ocular graduations coinciding with one graduation on the stage micrometer scale is determined.

A. Calibration of an ocular micrometer

Materials Required:

1. Microscope
2. Ocular micrometer
3. Stage micrometer
4. Oil immersion

Procedure :

1. Remove the eye piece (ocular) lens from your microscope, unscrew it and insert the ocular micrometer disc on the circular shelf (metal diaphragm) with the engraved side down into it.

2. Place the stage micrometer on the microscope stage and bring its scale in the microscope's field center under a sharp focus first using the low-power objective, and thereafter with high power and oil-immersion objectives.
3. Turn the ocular lens until the parallel lines of the ocular micrometer be parallel with those of the stage micrometer, that is, the graduations on both micrometers be superimposed on each other.
4. Count the number of divisions, in both the ocular and stage micrometers, between the two coinciding lines.
5. Finally calculate the calibration factor for one ocular division as follows:

$$\text{One ocular division (in µm)} = \frac{\text{No. of divisions on stage micrometer}}{\text{No. of divisions on ocular micrometer}} \times 10\cancel{0}$$

(c.f)

Example: If 3 divisions of the stage micrometer line up with 20 divisions of the ocular micrometer.

$$\therefore \text{Each ocular division} = \frac{3}{20} \times 10\ \mu\text{m} = 1.5\ \mu\text{m}$$

B. Measurement of Microorganisms by the use of an ocular micrometer

Materials Required:

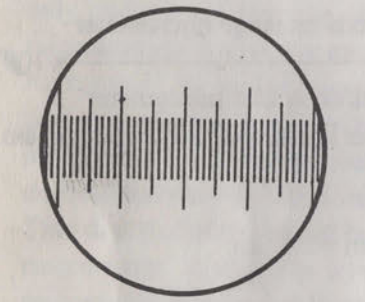
1. Ocular micrometer
2. Microscope
3. Slides of various organisms (yeast, bacteria)

Procedure:

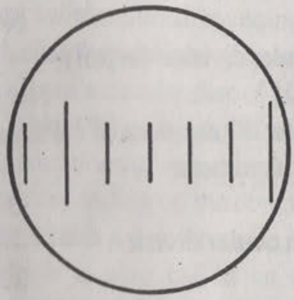
1. After calibration, replace the stage micrometer with the prepared slide of the microorganism.
2. Count the number of ocular micrometer divisions a single cell occupies.
3. By rotating the ocular lens, determine the divisions at least three readings each for length and width of the organism (if it is rod shaped) and at least three readings for diameter of the organism (if it is cocli shaped)
4. Determine the size of a microorganism by using following formula:

Size of organism = No. of ocular divisions occupied X calibration factor for one ocular division.

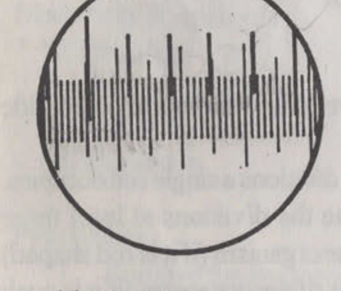
Example: If an organism occupied five ocular divisions, then,
The size of organism = 5 x 1.5 = 7.5 µm.
Note: Remove the ocular micrometer from your microscope at the end of the laboratory work.



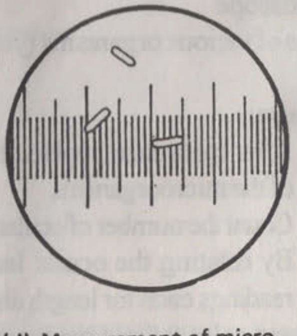
(a) Ocular micrometer:



(b) Stage micrometer:



(c) Superimposed ocular micrometer (below) over stage micrometer (above):

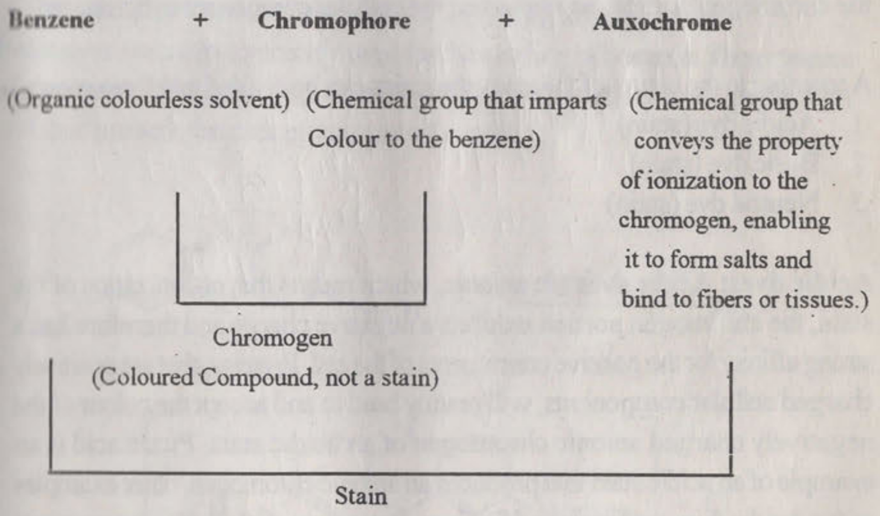


(d) Measurement of micro-organisms with the calibrated micrometer:

Section - III

The Staining of Microorganisms

It is difficult to observe microorganisms by our naked eyes because they are very minute and transparent, as well as they are colorless when they are suspended in an aqueous medium. An optical instrument i.e. microscope is used to observe the microorganisms. A better way of observing microorganisms like bacteria through the microscope is to prepare a slide by using different dyes (stains). This process is called the staining of microorganism. The staining procedures in conjunction with light microscopy is the tool for the study of properties of microorganisms and to divide microorganisms into specific groups for diagnostic purposes. There are different types of stains used for the staining purpose. Chemically a stain (dye) may be defined as an organic compound containing a benzene ring plus a chromophore and auxochrome group.



Benzene + **Chromophore** + **Auxochrome**

(Organic colourless solvent) (Chemical group that imparts Colour to the benzene) (Chemical group that conveys the property of ionization to the chromogen, enabling it to form salts and bind to fibers or tissues.)

Chromogen
(Coloured Compound, not a stain)

Stain

Figure 3.1 Chemical composition of a stain.

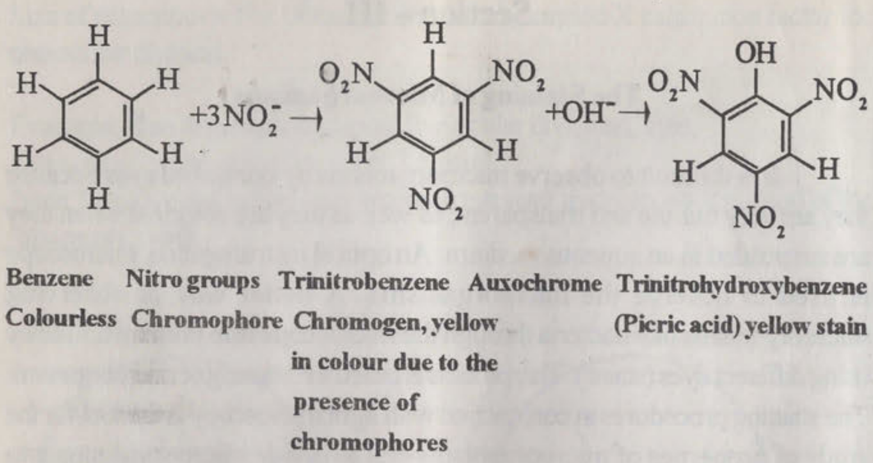

Benzene
Colourless
Nitro groups
Chromophore
Trinitrobenzene
Chromogen, yellow in colour due to the presence of chromophores
Auxochrome
Trinitrohydroxybenzene
(Picric acid) yellow stain
+3NO₂⁻
+OH⁻

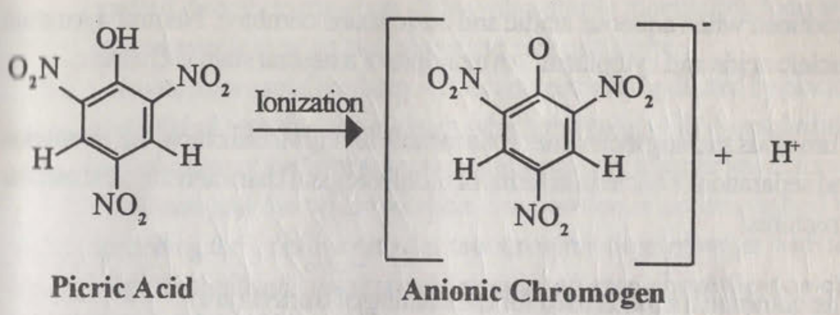
Figure 3.2 : The stain picric acid may be used as an example.

The ability of a stain to bind to macromolecular cellular components such as proteins or nucleic acids depends on the electrical charge found on the chromogen portion, as well as on the cellular component to be stained.

According to the nature of the stain, the stains can be divided into three groups:

1. Acidic dye (stain)
2. Basic dye (stain)
3. Neutral dye (stain)

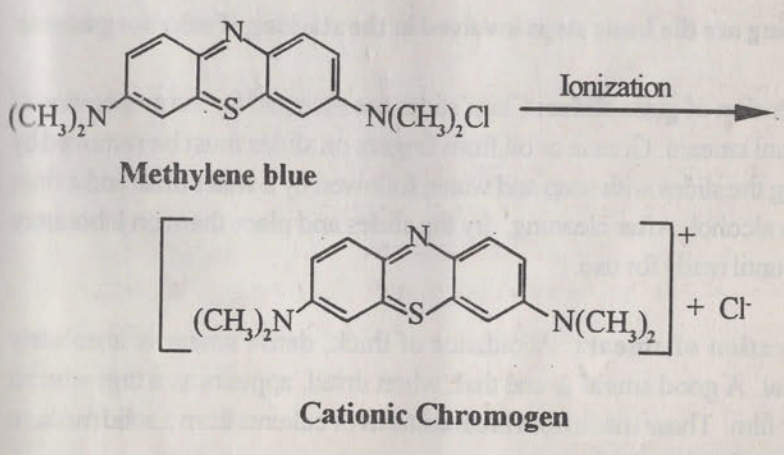
Acidic dyes: Acidic dyes are anionic, which means that on ionization of the stain, the chromogen portion exhibits a negative charge and therefore has a strong affinity for the positive constituents of the cell. Proteins, that are positively charged cellular components, will readily bind to and accept the colour of the negatively charged anionic chromogen of an acidic stain. Picric acid is an example of an acidic stain that produces an anionic chromogen, other examples of the Acidic dyes are Eosin and Sodium, Potassium, Calcium or Ammonium salts of coloured acids, that ionize to give a negatively charged chromogen. Acidic dyes are not very often used in microbiology laboratory except to provide background staining.


Ionization
Picric Acid
Anionic Chromogen
+ H⁺

Basic dyes: Basic dyes are cationic, because on ionization the chromogen portion exhibits a positive charge and therefore has a strong affinity for the negative constituents of the cell. Nucleic acids, negatively charged cellular components, will readily bind to and accept the colour of the positively charged, cationic chromogen of a basic stain.

Methylene blue is a basic stain that produces a cationic chromogen, other examples of the basic dyes are crystal violet, and the chloride or sulfate salts of coloured bases ionize to give a positively charged chromogen.

Basic dyes are more commonly used for the staining of bacteria. The presence of a negative charge on the bacterial surface, acts to repel most acidic dyes and thus prevent their penetration into the cell.


Ionization
Methylene blue
Cationic Chromogen
+ Cl⁻

Neutral dyes: Neutral stains are usually formed from the precipitate which is produced when aqueous acidic and basic stains combine. Neutral dyes stain nucleic acids and cytoplasm. An example of a neutral stain is Giemsa.

Numerous staining techniques are available for the visualization, differentiation and separation of bacteria in terms of morphological characteristics and cellular structures.

The common methods used for the staining of bacteria are:

1. **Simple staining**
2. **Differential staining**

1. Simple staining: For the simple staining, single stain is used. This staining technique is used for the visualization of morphological shape (cocci, bacilli and spirilli) and arrangement (chains, clusters, pairs and tetrads) of microbial cells.

2. Differential staining: For this staining technique, two or more contrasting stains are used. This technique is useful for the separation of microorganisms into groups eg: Gram stain and acid-fast stain, and for the visualization of structures, eg: Capsule stain, spore stain, flagella stain and Nuclear stain.

Following are the basic steps involved in the staining of microorganisms:

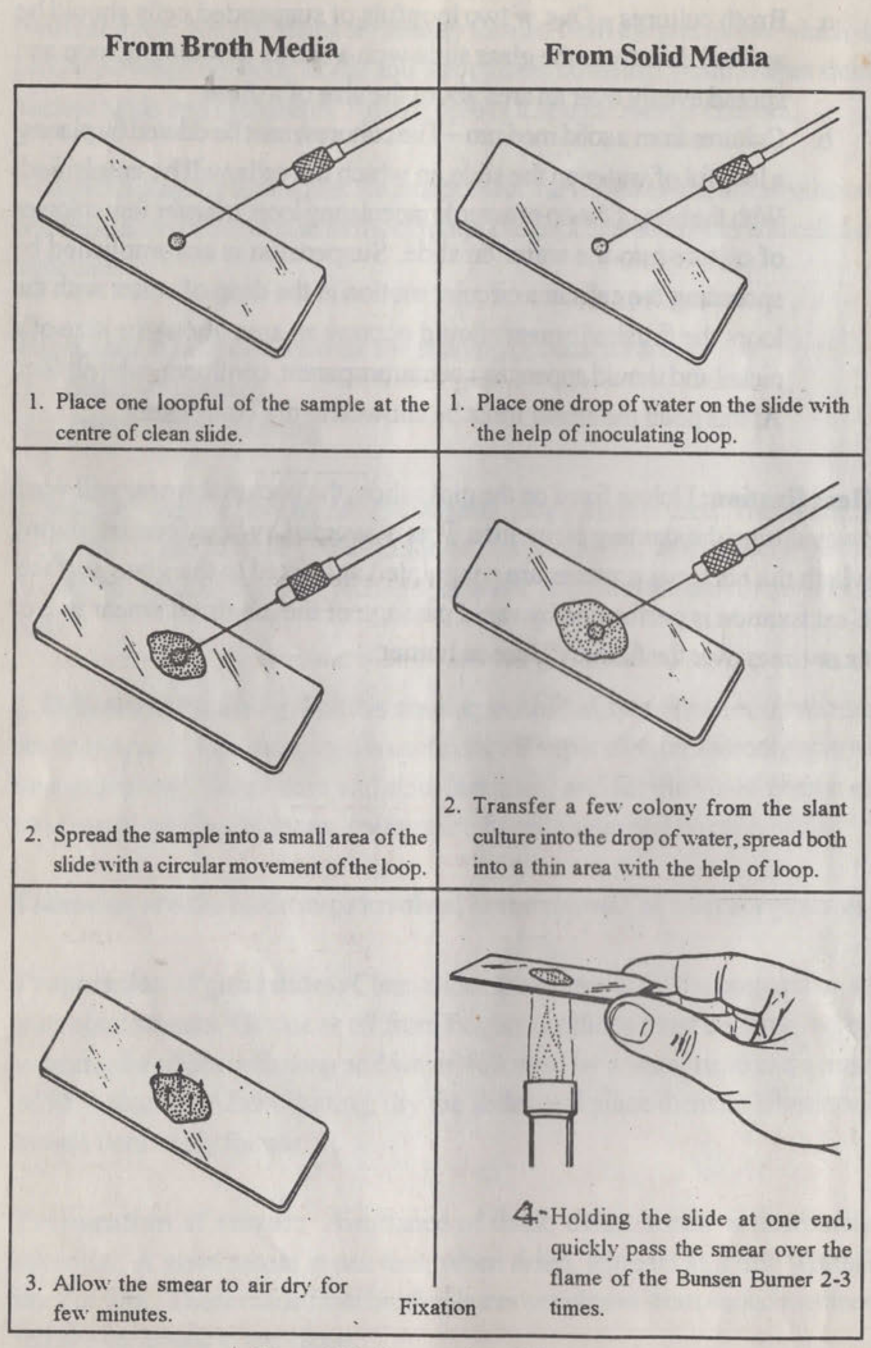
Preparation of glass slides: Clean slides are essential for the preparation of microbial smears. Grease or oil from fingers on slides must be removed by washing the slides with soap and water, followed by a water rinse and a rinse of 95% alcohol. After cleaning, dry the slides and place them on laboratory towels until ready for use.

Preparation of smear: Avoidance of thick, dense smear is absolutely essential. A good smear is one that, when dried, appears as a thin whitish layer or film. Those made from broth cultures or cultures from a solid medium require variations in technique.

a. Broth cultures – One or two loopfuls of suspended cells should be applied directly to the glass slide with a sterile inoculating loop and spread evenly over an area about the size of a dime.

b. Cultures from a solid medium – The cultures must be diluted by placing a loopful of water on the slide, in which the cells will be emulsified. With the help of the tip of a sterile inoculating loop, transfer few colonies of culture into the water on slide. Suspension is accomplished by spreading the cells in a circular motion in the drop of water with the loop. the finished smear should occupy an area about the size of a nickel and should appear as a semitransparent, confluent, whitish film. At this point the smear must be allowed to dry completely.

Heat fixation: Unless fixed on the glass slide, the bacterial smear will wash away during the staining procedure. This is avoided by heat fixation, during which the bacterial proteins are coagulated and fixed to the glass surface. Heat fixation is performed by rapid passage of the air-dried smear two or three times over the flame of Bunsen burner.


From Broth Media

From Solid Media

1. Place one loopful of the sample at the centre of clean slide.

1. Place one drop of water on the slide with the help of inoculating loop.

2. Spread the sample into a small area of the slide with a circular movement of the loop.

2. Transfer a few colony from the slant culture into the drop of water, spread both into a thin area with the help of loop.

3. Allow the smear to air dry for few minutes.

Fixation

4. Holding the slide at one end, quickly pass the smear over the flame of the Bunsen Burner 2-3 times.

Figure 3.3 : Preparation of Smear.

The common methods used for the staining of microorganisms are as follows:

- **i. Simple staining**
- **ii. Negative staining**
- **iii. Gram staining**
- **iv. Capsule staining**
- **v. Spore staining**
- **vi. Acid-fast staining**

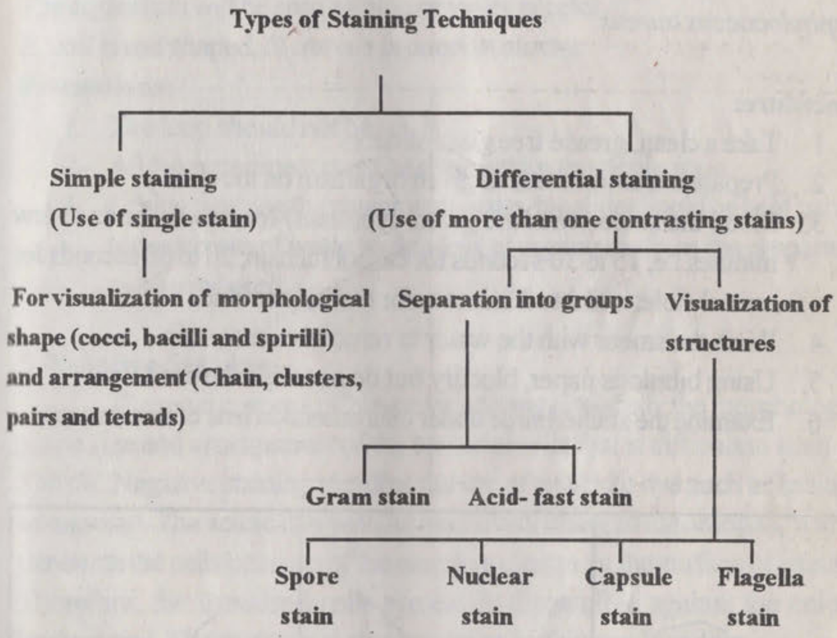

Types of Staining Techniques
Simple staining
(Use of single stain)
Differential staining
(Use of more than one contrasting stains)
For visualization of morphological shape (cocci, bacilli and spirilli) and arrangement (Chain, clusters, pairs and tetrads)
Separation into groups
Visualization of structures
Gram stain
Acid- fast stain
Spore stain
Nuclear stain
Capsule stain
Flagella stain

Figure 3.4: Different Staining Techniques.

4. Simple Staining

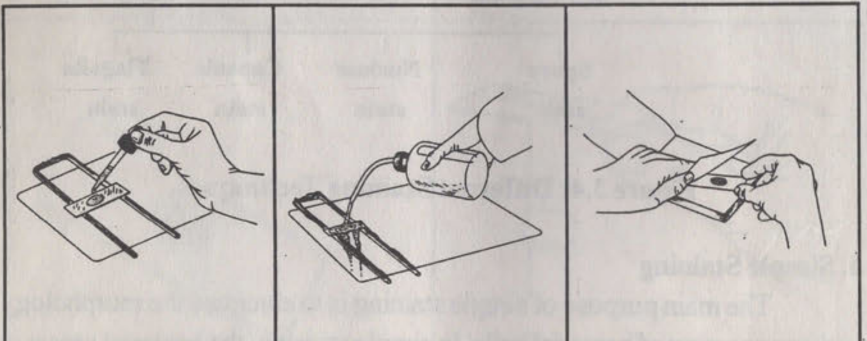
The main purpose of simple staining is to elucidate the morphology and arrangement of bacterial cells. In simple staining, the bacterial smear is stained with a single reagent. For this staining, basic stains with a positively charged chromogen are preferred because the bacterial cell components i.e, nucleic acids and certain cell wall components carry a negative charge that strongly attracts and binds to the cationic chromogen. The most commonly used basic stains are methylene blue, crystal violet and carbol fuchsin.

Materias Required

(1) Apparatus : Bunsen burner, glass slides, inoculating loop, staining tray, lens paper, bibulous paper and microscope.
(2) Reagents: Methylene blue, or crystal violet or carbol fuchsin.
(3) Cultures: 24 hours culture of *Escherichia coli, Bacillus cereus* and *Staphylococcus aureus.*

Procedure:

1. Take a clean, grease free glass slide.
2. Prepare bacterial smear of given organism on the slide.
3. Flood the smear with the given dye(stain) for few seconds to few minutes.i.e, 15 to 30 seconds for carbol fuchsin, 20 to 60 seconds for crystal violet and 1 to 2 minutes for methylene blue.
4. Wash the smear with the water to remove excess stain.
5. Using bibulous paper, blot dry but do not wipe the slide.
6. Examine the stained slide under oil immersion lens of microscope.



1. Stain a bacterial smear with crystal violet for one minute.
2. Wash briefly the smear with water.
3. Blot dry with Bibulous paper.

Figure 3.5: Procedure for Simple Staining.

Observation:

S.N.	Staining method	Stain used	Shape of cell	Colour of cell	Results

Results:
The organisms will be seen as blue or violet in color.
E. coli is rod shaped, *S. aureus* is cocci in cluster.

Precautions:

1. The loop should not be too hot.
2. All the experiment must be done within the sterile zone.
3. During washing the smear with water, the slide should be held parallel to the stream of water so that loss of organisms from the preparation can be reduced.

5. Negative Staining

The main purpose of Negative staining is to study the morphological shape size and arrangement of the bacterial cells that is difficult to stain e.g. *Spirilli.* Negative staining requires the use of an acidic dye such as India ink or nigrosin. The acidic dye with its negatively charged chromogen, will not penetrate the cells because of the negative charge on the surface of bacteria. Therefore, the unstained cells are easily discernible against the colored background. The methods is also known as background staining.
The practical application of negative staining is that, since heat fixation is not required and the cells are not subjected to the distorting effects of chemicals and heat, their natural shape and size can be seen.

Materials Required
Apparatus:

1. Bunsen burner,
2. Inoculating loop,

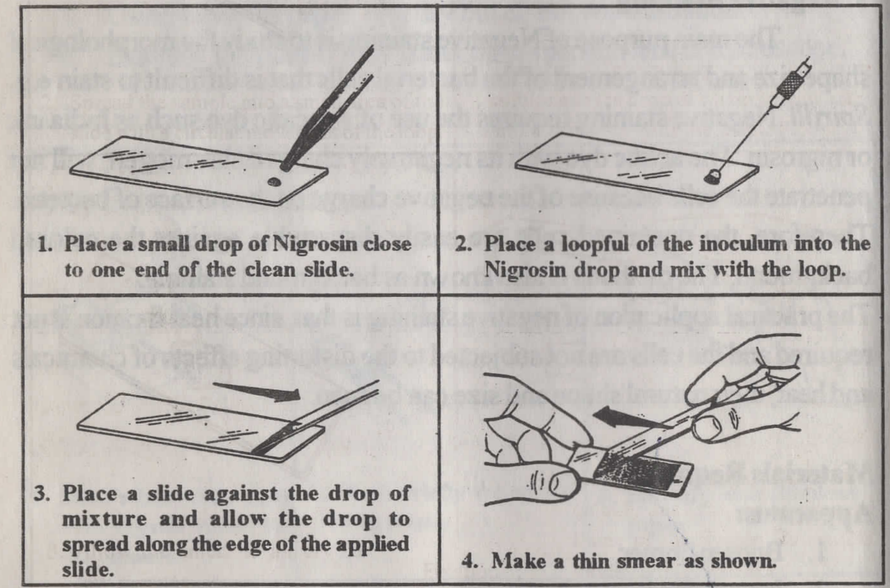
3. Glass slides
4. Staining tray
5. Lens paper
6. Bibulous paper
7. Microscope

Reagent: Indian ink or Nigrosin.

Culture: 24 hours agar slant cultures of *Bacillus cereus, Micrococcus luteus*

Procedure:

1. Place a small drop of nigrosin, (not over 1/8" diameter), close to one end of a clean slide (about 3/4" from one end of slide).
2. Aseptically, place one loopful of inoculum from the given culture in the drop of nigrosin.
3. Make a thin smear with the help of another slide (held at a 30° angle) by sliding it over the slide containing the sample.
4. Air dry. Do not heat fix the slide.
5. Examine the slide under microscope, first in low power and then in oil-immersion.



1. Place a small drop of Nigrosin close to one end of the clean slide.

2. Place a loopful of the inoculum into the Nigrosin drop and mix with the loop.

3. Place a slide against the drop of mixture and allow the drop to spread along the edge of the applied slide.

4. Make a thin smear as shown.

Figure 3.6 : Procedure for Negative Staining.

Observation:

S.N.	Staining Method	Stain used	Colour of the organism	Shape of the organism	Results
	Negative staining	Nigrosin			

Results:

1. The bacterial cells will be seen transparent with blue background.
2. *Bacillus cereus* will be seen rod shaped and *Micrococcus luteus* will be seen cocci shaped.

Precautions:

1. The slide must be absolutely clean: the presence of any residual grease or dirt on the slide will produce an uneven smear.
2. The loop should not be too hot.
3. The amount of nigrosine or India ink is critical.
4. Heat fixation is not necessary as heat destroy the shape of organism.

6. Gram Staining

Gram staining is one of the most important and widely used differential staining technique in microbiology, which was introduced, for the first time, by the Danish bacteriologist Hans Christain Gram in 1884. The main purpose of the gram stain is to differentiate the bacteria between the two major groups gram positive and gram negative, which makes it an essential tool for classification and differentiation of microorganisms. In case of bacterial cell, pores are present, through which stains enter and give its color. In this technique, the fixed bacterial smear is subjected to the following staining reagents in the order listed below:

Primary Stain

Crystal Violet: Crystal violet stain is used first and stains all cells purple

Mordant:

Gram's Iodine: It serves as a mordant, a substance that forms an insoluble complex by binding to the primary stain. The resultant Crystal violet – Iodine (CV-I) complex serves to intensify the color of the stain, and all the cells will appear purple-black at this point. In Gram positive cells only, this CV-I complex binds to the magnesium-ribonucleic acid component of the cell wall. The resultant magnesium-ribonucleic acid-crystal violet-iodine (Mg-RNA-CV-I) complex is more difficult to remove than the smaller CV-I complex.

Decolourizing Agent:

Ethyl alcohol (95%): This reagent serves a dual function as a lipid solvent and as a protein-dehydrating agent. Its action is determined by the lipid concentration of the microbial cell walls. In gram positive cells, the low lipid concentration is important to retention of the Mg-RNA-CV-I complex. Therefore, the small amount of lipid content is readily dissolved by the action of the alcohol, causing formation of minute cell wall pores. These are then closed by the alcohol's dehydrating effect. Consequently, the tightly bound primary stain is difficult to remove, and the cells remain purple. In Gram negative cells, the high lipid concentration found in outer layers of the cell wall is dissolved by the alcohol, creating large pores in the cell wall that do not close appreciably on dehydration of cell wall proteins. This facilitates release of the unbound CV-I complex, leaving these cells colourless or unstained.

Counterstain:

Safranin: This is the final reagent, used to stain red those cells that have been previously decolourized. Since only Gram negative cells undergo decolourization, they may now absorb the counterstain. Gram positive cells retain the purple colour of the primary stain.

In case of bacterial cells, pores are present through which stain enter and gives its color. According to gram's staining method, bacteria are of two

Table 3.1: Some characteristics of Gram-positive and Gram-negative Bacteria and Examples of some Important Pathogenes.

Characteristics	Gram-positive		Gram-negative	
Gram's reaction (Microscopic appearance)	Dark-violet or purple		Red (i.e., colour of the counterstain)	
2. Cell-wall composition	Low in lipids (1-4%)		High in lipids (11-22%)	
3. Susceptibility to penicillin	More susceptible		Less susceptible	
4. Effect of basic dyes (e.g. crystal violet)	Marked inhibition		Less inhibition	
5. Resistance to physical disruption	More resistant		Less resistant	
	Cocci	Bacilli	Cocci	Bacilli
	Streptococci mutans	*Corynebacterium diptheriae*	*Neisseria gonorrhoeae*	*Escherichia coli*
	Streptococcus pyogens	*Mycobacterium leprae*	*Neisseria meningitidis*	*Salmonella typhosa*
	Steptococcus faecalis	*Mycobacterium tuberculosis*		
	Staphylococus aureus	*Clostridium botulinum*		*Klebsiella pneumoniae*
	S. epidermidis	*Clostridium perfringens*		*Shigella dysenteriae*
		Clostridium novyti		*Shigella paradysenteriae*
		Bacillus anthracis		*Hemophilus ducreyi*
				Pseudomonas asruginosa
				Pasteurella tularensis
				Brucella melitensis
				Brucella abortus

types: Gram positive bacteria which retain crystal violet hence appear deep violet in colour and Gram negative bacteria which loses crystal violet colour and are counter stained by safranin and hence appear red in colour.

Materials Requried:

Apparatus:

1. Bunsen burner,
2. Inoculating loop,
3. Glass slides
4. Staining tray
5. Bibulous paper
6. Microscope.

Reagents:

1. Crystal violet
2. Gram's Iodine
3. 95% Ethyl alcohol
4. Safranin

Cultures:

24 hours nutrient agar slant cultures of *Escherichia coli, Staphylococcus aureus*, and *Bacillus cereus*.

Procedure: -

1. Take a clean, grease free slide.
2. Prepare the smear of suspension on slide with a loopful of sample then air dry and heat fix the smear.
3. Flood the smear with crystal violet for 30 seconds to 1 minute.
4. Wash the smear with water.
5. Flood the smear with the gram's iodine and let stand for 1 minute.
6. Wash with water.
7. Decolorize with decolorizer, 95% ethyl alcohol for 10-20 seconds, add reagent drop by drop until crystal violet fails to wash from smear.
8. Wash the slide with water.
9. Counterstain with safranin for 45 seconds.
10. Wash with water.
11. Blot dry with bibulous paper and examine under oil immersion.

Table summarizes the entire process.

Table 3.2: Summary of the Entire Process.

Reagent	Time	Colour	
		Gram Positive	Gram Negative
Primary stain: Crystal violet	30 sec. to 1 min.	Purple	Purple
Mordant: Gram's Iodine	1 minute	Purple	Purple
Decolourizing agent: 95% alcohol	10-20 seconds	Purple	Colourless
Counterstain : Safranin	45 seconds	Purple	Pink

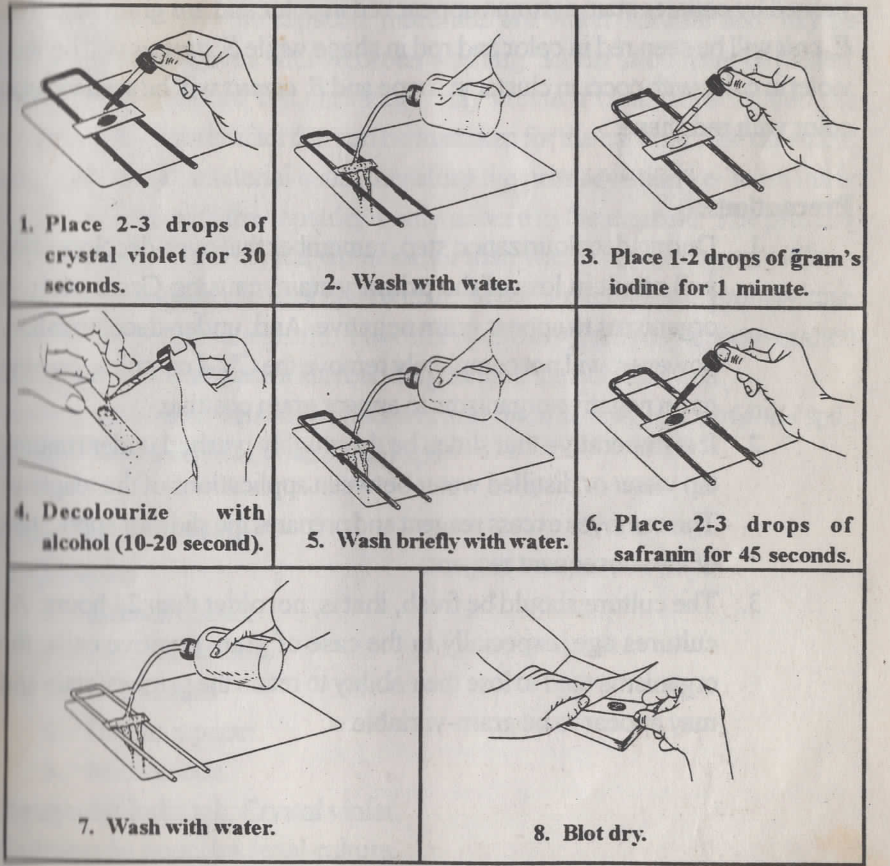

1. Place 2-3 drops of crystal violet for 30 seconds.
2. Wash with water.
3. Place 1-2 drops of gram's iodine for 1 minute.
4. Decolourize with alcohol (10-20 second).
5. Wash briefly with water.
6. Place 2-3 drops of safranin for 45 seconds.
7. Wash with water.
8. Blot dry.

Figure 3.7 : Procedure for Gram Staining.

Observation

S.N.	Staining method	Stain used	Colour of organism	Shape of organism	Results
	Gram staining	Crystal violet, Iodine,Alcohol safranin.			

Results: Bacterial cells which retain crystal violet appear deep violet in color and are gram positive whereas bacterial cells that loses violet color and are stained by counter stain safranin appear red in color and are gram negative. *E. coli* will be seen red in color and rod in shape while *S. aureus* will be seen violet in color with cocci in cluster in shape and *B. cereus* will be seen violet in color with rod shape.

Precautions:

1. During decolourization step, remember that over-decolorization will result in loss of the primary stain, causing Gram positive organisms to appear gram negative. And, under-decolorization, however, will not completely remove the CV-I complex, causing gram negative organisms to appear gram positive.
2. It is imperative that slides be thoroughly washed under running tap water or distilled water between applications of the reagents. This removes excess reagent and prepares the slide for application of the subsequent reagent.
3. The culture should be fresh, that is, not older than 24 hours. As cultures age, especially in the case of gram positive cells, the organisms tend to lose their ability to retain the primary stain and may appear to be gram-variable.

7. Capsule Staining

The main purpose of capsule stain is to distinguish capsular material from the bacterial cell. A Capsule is a gelatinous outer layer secreted by bacterial cell and that surrounds and adheres to the cell wall. It is not common to all organisms. Cells that have a heavy capsule are generally virulent and capable of producing disease, since the structure protects bacteria against the normal phagocytic activities of host cells and the attachment of bacteriophage. It also helps to attach the bacteria to the host cell. Chemically, capsule material is a polysaccharide, a glycoprotein, or a polypeptide.

Capsule staining is more difficult than other types of differential staining procedure because the capsular materials are water-soluble and may be dislodged and removed with vigorous washing. Smear should not be heated, because the resultant cell shrinkage may create a clear zone around the organism that is an artifact that can be mistaken for the capsule. The capsule is non-ionic unlike bacterial cells, therefore the primary stain i.e. India ink is unable to bind with the capsule, it only adhere to the capsule. The primary stain is washed with crystal violet rather than water. As the crystal violet is basic dye so the bacterial cell bind with it. At the same time, the primary stain is removed from the capsule. Therefore, under microscopic examination capsule is seen transparent surrounding around the bacterial cell.

Some examples of capsulated bacteria are: *Bacillus* sps., *Clostridium* sps., *Klebsiella pneumonia, Rhizobium* sps.

Materials Required:

Apparatus:

1. Bunsen burner,
2. Inoculating loop,
3. Glass slides
4. Bibulous paper
5. Microscope.

Reagents: India ink, Crystal violet.

Culture: 24 hours bacterial culture.

Procedure:

1. Take a clean, grease free slide.
2. Using sterile technique, prepare a heavy smear of the culture.
3. Allow smear to air dry.
4. Flood smear with India ink and let stand for 5-7 minutes.
5. Wash the smear with crystal violet solution for one minute.
6. Gently dry by blotting and examine under microscope first at low power and then at oil immersion.

Observation:

S.N.	Staining method	Stain used	Colour of organism	Colour of capsule	Results
	Capsule staining	India Ink Crystal violet			

Results: Under microscopic examination the capsule of the bacterial cell will be seen transparent with deep blue background.

Precautions:

1. The slide must be clean and grease free.
2. Heat fixation must not be done.
3. All the experiment must be done in the sterile zone.

8. Acid-Fast Staining (Ziehl-Neelsen Method)

The main purpose of Acid-Fast stain is to differentiate bacteria into acid-fast and non acid-fast groups. While the majority of bacterial organisms are stainable by either simple or gram staining procedure, a few genera, particularly the members of the genus *Mycobacterium,* are resistant and can only be visualized by the acid fast method. Since *M. tuberculosis* and *M. leprae* represent bacteria that are pathogenic to humans, the stain is of diagnostic value in identifying these organisms.

The characteristic difference between *Mycobacteria* and other microorganisms is the presence of a thick waxy (lipoidal) wall that makes penetration by stains extremely difficult. Once the stain has penetrated, however, it cannot readily be removed even with the vigorous use of acid alcohol as a decolorizing agent. Because of this property, these microorganisms are called **acid-fast**, while all other microorganisms, which are easily decolorized by acid-alcohol, are called **non-acid-fast**.

The acid-fast stain uses three different reagents.

Primary stain:

Carbol Fuchsin: This is used as a primary stain, Carbol Fuchsin, a red phenolic stain is soluble in the lipoidal materials that constitute the major portion of the Mycobacterial cell wall. It does penetrate these bacteria and is retained. Penetration is further enhanced by the application of heat, which drives the carbol fuchsin through the lipoidal wall and into the cytoplasm. After application of the primary stain all cells appear red.

Decolorizing Agent:

Acid-Alcohol (Hcl+95%Ethanol): On application of acid-alcohol, acid-fast cells will be resistant to decolorization, since the primary stain is more soluble in the cellular waxes than the decolorizing reagent. In this event, the primary stain is retained and the *Mycobacteria* will stay red. This is not the case with the non-acid-fast organisms that lack the cellular waxes in their cell wall .The primary stain is more easily removed during decolorization, leaving these cells colourless or unstained.

Counterstain:

Methylene blue: This is used as the final reagent to stain previously decolorized cells. As only non-acid-fast cells undergo decolorization, they may now absorb the counterstain and take on its blue color, while acid-fast cells retain the red color of the primary stain.

Materials Required:

Apparatus:

1. Bunsen Burner,
2. Water bath,
3. Inoculating loop,

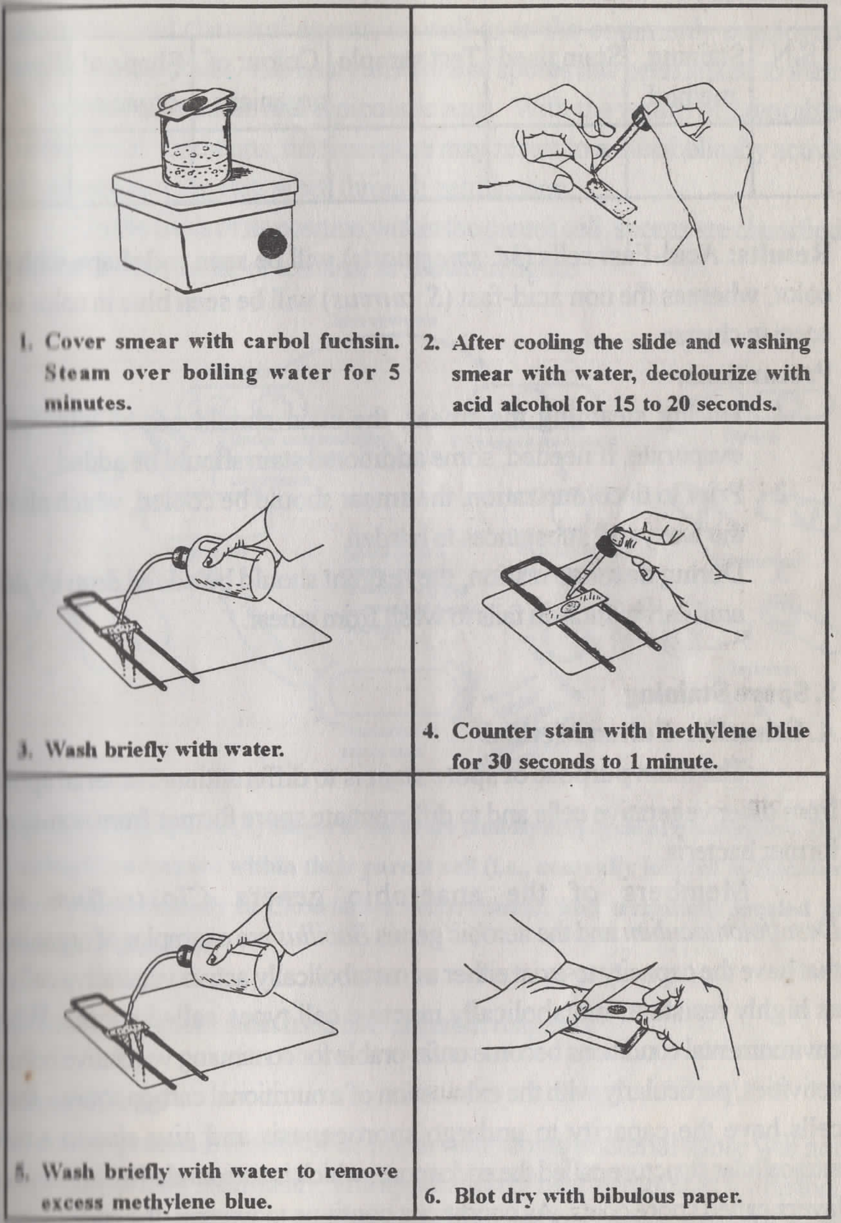
4. Glass slides
5. Bibulous paper
6. Staining tray
7. Microscope.

Reagents:
Carbol fuchsin,
Acid-alcohol, and
Methylene blue.

Cultures: 72 hours trypticase soy broth culture of *Mycobacterium smegmatis* and 18-24 hours culture of *Staphylococcus aureus*.

Procedure:

1. Take a clean and grease free glass slide.
2. Using sterile technique, prepare a bacterial smear on slide.
3. Allow smears to air-dry then heat fix in the usual manner.
4. Flood smear with carbol fuchsin and place on a water bath allowing the preparation to steam for 5 minutes
5. Wash with water. Heated slides must be cooled before washing.
6. Decolourize with acid-alcohol, adding the reagent drop by drop
7. Wash with water.
8. Counter stain with methylene blue for 30 second to 1 minute.
9. Wash smear with water.
10. Blot dry with bibulous paper and examine under oil immersion.



1. Cover smear with carbol fuchsin. Steam over boiling water for 5 minutes.

2. After cooling the slide and washing smear with water, decolourize with acid alcohol for 15 to 20 seconds.

3. Wash briefly with water.

4. Counter stain with methylene blue for 30 seconds to 1 minute.

5. Wash briefly with water to remove excess methylene blue.

6. Blot dry with bibulous paper.

Figure 3.8 : Procedure for Ziehl-Neelsen Acid-Fast Staining.

Observation:

S.N.	Staining method	Stain used	Test sample	Colour of organism	Shape of organism	Results

Results: Acid-Fast cells (*M. smegmatis*) will be seen rod shape with red color, whereas the non acid-fast (*S. aureus*) will be seen blue in color with cocci in cluster.

Precautions:

1. During steaming the smear, the stain should not be allowed to evaporate. If needed, some additional stain should be added.
2. Prior to decolourization, the smear should be cooled, which allows the waxy cell substances to harden.
3. During decolourization, the reagent should be added drop by drop until carbol fuchsin fails to wash from smear.

9. Spore Staining

A. Schaeffer-Fulton Method

The main purpose of spore stain is to differentiate bacterial spores from other vegetative cells and to differentiate spore former from non-spore former bacteria.

Members of the anaerobic genera *Clostridium* and *Desulfotomaculum* and the aerobic genus *Bacillus* are examples of organisms that have the capacity to exist either as metabolically active vegetative cells or as highly resistant, metabolically inactive cell types called spores. When environmental conditions become unfavorable for continuing vegetative cellular activities, particularly with the exhaustion of a nutritional carbon source, these cells have the capacity to undergo sporogenesis and give rise to a new intracellular structure called the endospore, which is surrounded by impervious layers called spore coats. As conditions continue to worsen, the endospore is released from the degenerating vegetative cell and becomes an independent cell called a spore. Because of the chemical composition of spore layers, the spore is resistant to the deleterious effects of excessive heat, freezing, radiation, desiccation, and chemical agents, as well as to the commonly employed microbiological stains. The heat resistance of spores has been linked to their high content of calcium and dipicolinic acid. With the return of favorable environmental conditions, the free spore may revert to a metabolically active and less resistant vegetative cell through germination.

On the basis of its position within the parent cell, spores are classified as central, subterminal, or terminal as shown in figure.

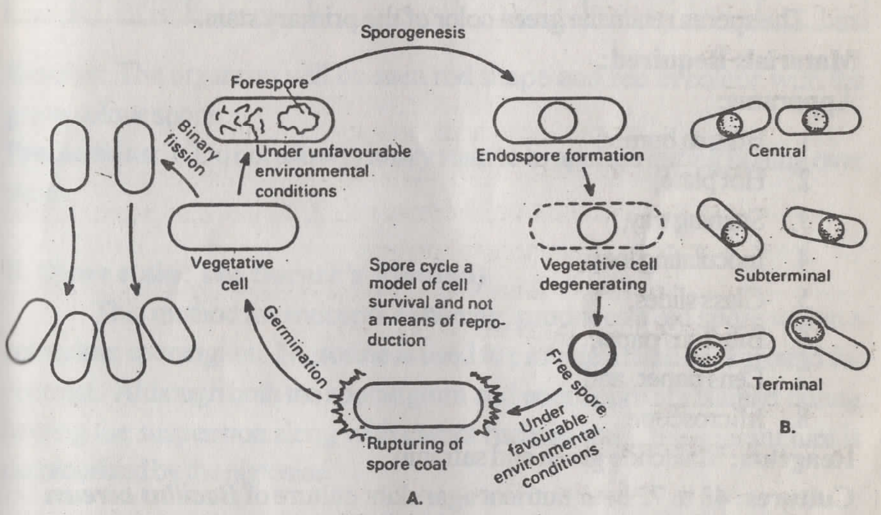

Sporogenesis
Forespore
Binary fission
Under unfavourable environmental conditions
Endospore formation
Central
Vegetative cell
Spore cycle a model of cell survival and not a means of reproduction
Vegetative cell degenerating
Subterminal
Germination
Free spore
Terminal
Under favourable environmental conditions
B.
Rupturing of spore coat
A.

Figure 3.9 : Endospores (A) Stages in the spore (endospore) cycle of a bacterium. (B) Location of endospore within their parent cell (i.e., centrally located in *Bacillus cereus*, substerminally in *Clostridium subterminale*, and terminally located in *Clostridium tetani*).

In practice, the spore stain uses two different reagents.

Primary Stain:

Malachite green: Because of its impervious coats bacterial spore will not accept the primary stain easily. The application of heat is required for further penetration. After the primary stain is applied and the smear is heated, both the vegetative cell and spore will appear green.

Decolorizing Agent:

Distilled water: Once the spore accepts the malachite green, it can not be decolorized by water, which removes only the excess primary stain. The spore will remain green. On the other hand, the stain does not demonstrate a strong affinity for vegetative cell components, and the water removes the malachite green. So that the vegetative cell becomes colourless.

Counter stain:

Safranin: This contrasting red stain is used as the second reagent to colour the decolorized vegetative cells, which will absorb the counter stain and appear red. The spores retain the green color of the primary stain.

Materials Required:

Apparatus:

1. Bunsen burner,
2. Hot plate,
3. Staining tray,
4. Inoculating loop,
5. Glass slides,
6. Bibulous paper,
7. Lens paper, and
8. Microscope.

Reagents: Malachite green and safranin

Cultures: 48 to 72 hour nutrient agar slant culture of ***Bacillus cereus***:

Procedure:

1. Take a clean, grease free glass slide.
2. Make smear using sterile technique.
3. Allow smear to air dry and heat fix.
4. Flood smear with malachite green and steam over boiling water for 5 minutes. Add additional stain if stain boils off.
5. Remove slide from boiling water, cool, and wash with water.
6. Counter stain with safranin for 20-30 second,
7. Wash with water to remove safranin.
8. Blot dry with bibulous paper and examine under oil immersion.

Observation:

S.N.	Staining method	Stain used	Colour of organism	Colour of spores	Location of spores	Results

Results: The organism will be seen rod shape and red in colour with the green colour spore.

Precautions: 1. Do not allow primary stain to evaporate during boiling over steam.

B. Spore stain (The Dorner's Method)

This method for endospore staining, produces a red spore within a colourless sporangium. Nigrosine is used to provide a dark background for contrast. Although both the sporangium and endospore are stained during heating the suspension along with carbol fuchsin stain, the sporangium is decolourized by the nigrosine.

Materials Required:

Apparatus:

Electric hot plate, small beaker, small test tubes, inoculating loop, glass slides, microscope.

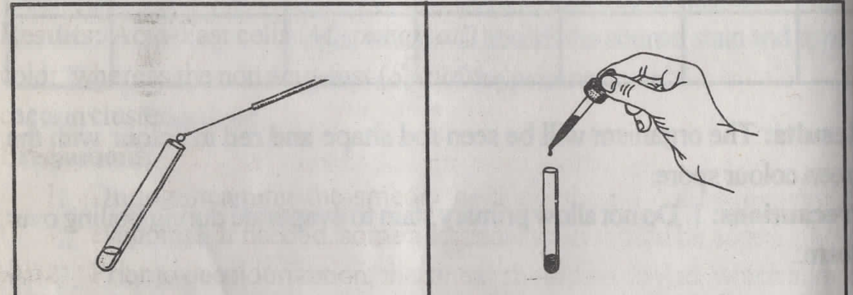
Reagents : Carbol-fuchsin stain
Nigrosine

Cultures: 24-48 hours nutrient agar slant culture of *Bacillus megaterium*.

Procedure:

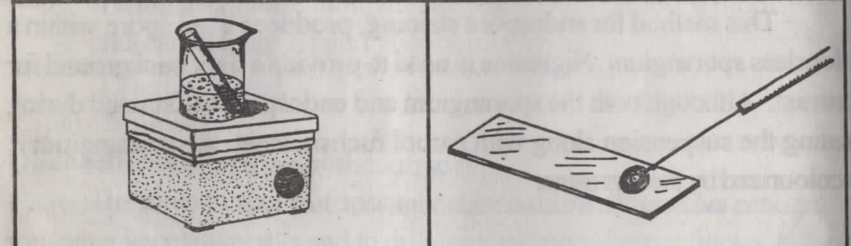
1. Make a heavy suspension of bacteria by dispersing several loopfuls of bacteria in 5 drops of sterile water in a test tube.
2. Add 5 drops of carbol-fuchsin to bacterial suspension.

3. Heat carbol-fuchsin suspension of bacteria in a beaker of boiling water for 10 minutes.
4. Mix several loopfuls of bacteria in a drop of nigrosine on a slide.
5. Spread nigrosine-bacteria mixture on the slide with the help of an another spreader slide.
6. Allow smear to air dry.
7. Examine the slide under oil immersion lens.



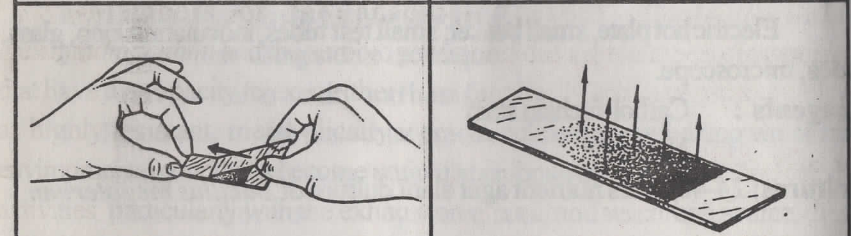
1. Make a heavy suspension of bacteria in 5 drops of sterile water in a tube.

2. Add 5 drops of carbol fuchsin to the bacterial suspension.



3. Heat the mixture in a beaker of boiling water for 10 minutes.

4. Mix 3-4 loopfuls of bacteria in a drop of Nigrosine on the slide.



5. Spread the Nigrosine-bacteria mixture on the slide with the help of an another spreader slide.

6. Allow the smear to air dry and examine under oil immersion.

Figure 3.10 : Procedure for Spore Staining (The Dorner's Method)

Section - IV

10. Motility Determination

For the identification of an unknown organism it is often necessary to determine whether or not the microorganism is motile. Most of the rod shaped bacteria are motile. There are several ways that one can use to make this determination. The three methods used for motility determination are (i) the wet mount method (ii) the hanging drop method and (iii) a culture method. Each method has its advantages and disadvantages.

I. The Wet Mount

This is simplest method to determine the motility in which a few loopfuls of the organism is placed on a clean slide and covered with a cover slip. Bacterial movement must be distinguished from "Brownian movement". Brownian movement is vibratory movement caused by invisible molecules striking the bacteria, displacing them for short distances. True motility of the bacteria may lead to the bacteria to move from one place to other relatively longer distance.

The advantage of a wet mount is that it is the quickest means for determining motility. Such a preparation also is useful for determining cellular shape and arrangement. Whether or not the cells are arranged in irregular clusters, packets, pairs or long chains is more readily determined by this method than by staining because heat drying of a smear tends to cause organisms to coalesce.

One disadvantage of this method is that the slide quickly dries out rendering the organisms immotile. Another disadvantage is that if the organism is pathogenic, there is the possibility of danger to the technician in handling viable organisms on a slide.

Materials Required:

1. Glass slide
2. Cover slips
3. Inoculating loop
4. Microscope
5. Bunsen burner
6. Fresh broth culture of *Proteus species*

Procedure:

1. Transfer 4-5 loopfuls of sample on the centre of clean glass slide.
2. Handle the cover slip by its edges and place it on the drop.
3. Press the cover slip gently with the forcep.

Observation and Results

Observe the shape and characteristics of motility of bacteria.

S.N.	Sample	Colour	Shape	Movement	Results

ii. The Hanging drop Method

The hanging drop method is used when it is necessary to study viable organisms on a microscope slide for a longer period of time than is possible with a wet mount method. In this method, organisms are observed in a drop that is suspended under a cover slip in a concave slide (**cavity slide**). Since the drop lies within an enclosed glass chamber, drying out occurs very slowly. Hanging drop slides yield the same information as wet mount slides : motility information, cell shape and cell arrangement.

Materials Required.

1. Cavity slide
2. Cover slips
3. Vaseline/petroleum jelly
4. Match sticks
5. Culture of test organism.

Procedure:

1. Clean and flame a hanging-drop slide and place it on the table with the depression uppermost.
2. Spread a little Vaseline or petroleum jelly around the cavity of the slide.

3. Clean a cover slip and apply Vaseline on each of the four corners of it using a match stick.
4. Place the cover slip on a clean paper with the Vaseline side up.
5. Transfer one loopful of culture in the centre of the cover slip.
6. Place the depression slide on to the cover slip, with the cavity facing down so that the depression covers the suspension.
7. Press the slide gently to form a seal between the cover slip and the slide.
8. Lift the preparation and quickly turn the hanging drop preparation cover slip up so that the culture drop is suspended.
9. Examine the preparation under low-power objective with reduced light.
10. Switch to the high-power objective and examine the preparation again.
11. Place a drop of oil on the cover slip and examine the preparation under oil-immersion objectives.

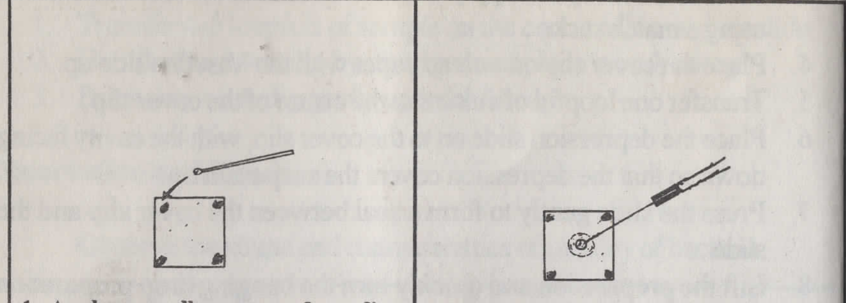
Observation : Observe the shape, colour and motility of the bacteria as :

S.No.	Sample	Colour	Shape	Movement	Results

Result : True motility will be shown by bacteria that move swiftly across the microscope.

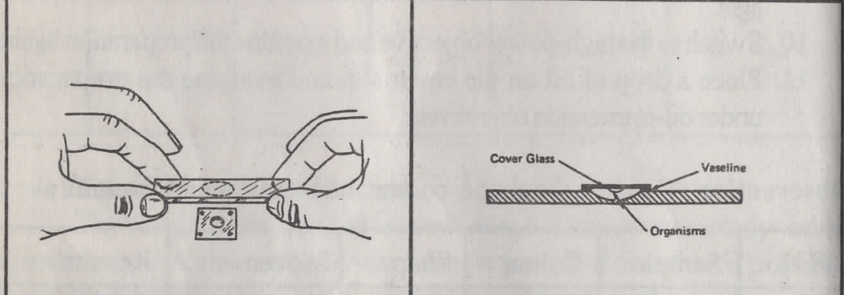
Precautions

1. The depression slide is inverted over the cover slip in such a way that the suspension does not touch the surface of the concavity at any point.
2. The slide and cover slip should be sterilized after the examination is finished.



1. Apply a small amount of vaseline on each of the four corners of the cover slip with a match stick.

2. Place 2 loopfuls of suspension in the centre of the cover slip.



3. Press the depression slide against vaseline on cover slip and invert quickly.

4. Examine the preparation under oil immersion.

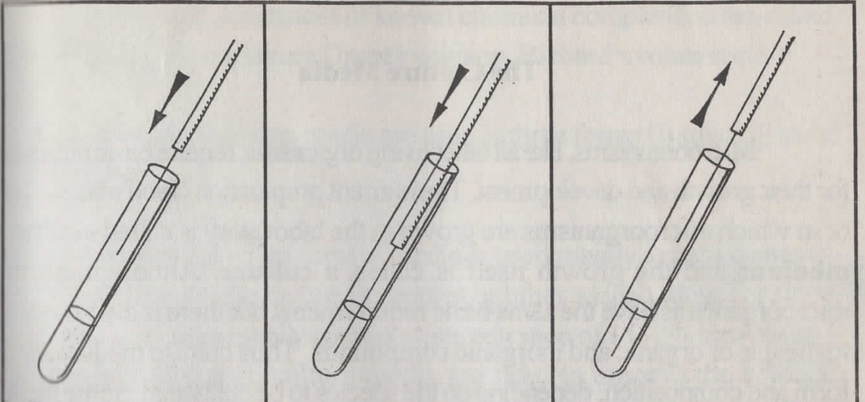
Figure 4.1 : Procedure for Motility (Hanging Drop Slide Method)

iii. A culture method

For pathogenic microorganisms such as typhoid bacillus, it is too dangerous to attempt to determine motility with slide techniques. A much safer method is to culture the organisms in a semisolid medium by the stab technique and observe the growth and motility.

Materials Required:

1. Inoculating wire
2. Bunsen burner
3. Tubes of semisolid medium
4. 24 hours broth culture of *Proteus sps.*



1. Bring the wire with organism into tube without touching walls of tube.

2. Penetrate the wire in the medium to 2/3 of its depth..

3. Withdraw the wire from medium and tube. Flame the neck of tubeand plug.

Figure 4.2 : Procedure for Stap Technique Motility Test.

Procedure:

1. Label the tube of semisolid medium with the name of organism.
2. Sterilize the inoculating wire by flaming and cool it for a while.
3. Take the suspension with wire from the culture tube.
4. Remove the plug from the tube of semisolid medium, flame the mouth and stab medium with inoculating wire containing test culture.
5. Flame the neck of the tube. Plug with the cotton.
6. Incubate the tube at 37°C for 24 to 48 hours in an incubator.
7. Observe the tube.

Observations: After incubation, examine the tube for cloudiness.

Results: If the organism is motile, a cloudiness will exist around the stab due to the migration of motile bacteria from the line of inoculation. Non-motile organisms grow only on the line of the stab.

Precaution:

1. The inoculation should be performed with a straight wire that is stabbed two-thirds of the way down into the medium.

Section - V

The Culture Media

Microorganisms, like all other living organisms, require basic nutrients, for their growth and development. The nutrient preparation (food material) on or in which microorganisms are grown in the laboratory is called a **culture medium** and the growth itself is called a **culture**. Although, all the microorganisms have the same basic requirements but there is a diversity as to the use of organic and inorganic compounds. Thus culture media vary in form and composition, depending on the species to be cultivated. Some media contain solutions of inorganic salts and may be supplemented with one or more organic compounds while other media contain complex ingredients such as extracts or digests of plant and animal tissues.

For the cultivation of virus and bacteriophages, living hosts are required. Bacteriophages propagate within certain bacterial cells, where the bacterial cell serves as the medium host for the bacteriophage.

Media have been classified variously using different criteria like chemical composition, physical state and utility purpose. On the basis of their composition, there are three main types of culture media:

i. Natural or empirical culture media.
ii. Semi-synthetic culture media and
iii. Synthetic or chemically defined culture media.

Natural or empirical culture media : Those media whose exact chemical composition is not known are called natural or empirical culture media. These include milk, urine, diluted blood, vegetable juices, meat extracts and infusions, peptone (digested protein rich in amino acids that provide nitrogen and carbon source).

Semi-synthetic culture media : Those media whose chemical composition is partially known are called semi-synthetic media. Any medium which contains agar becomes a semi-synthetic medium. Examples are Potato Dextrose agar, Czapek-Dox agar etc.

Synthetic or a chemically defined culture media : Those media which are composed of special substances of known chemical composition are called synthetic media. Examples are Czapek solution, Richard's solution etc.

On the basis of physical sate, media are used in three forms (i) solid (ii) semi solid (iii) Fluid.

Solid culture media : This form of media is used mainly in petri dishes as plate cultures. It can also be used in bottles or tubes as stab (deep) or slope cultures. These media usually contain a concentration of 1.0-2% agar to yield a suitable gel. The solid culture media are used to observe the colonial appearance, shape, size of colony, and the changes in the surrounding medium which help to identify the bacteria and differentiate commensals from Pathogens. Examples are Nutrient agar, Blood agar, Chocolate agar etc.

Semisolid Culture Media : This form of medium is prepared by adding a small amount of agar (0.2-0.5% W/V) to a fluid medium. Semi solid media are used mainly as transport media and for motility testing. Examples are, Amies transport medium, Cary-Blair transport medium etc.

Fluid culture media : This form of media do not contain agar or any solidifying agent. Growth of microorganims in these media is shown by a turbidity in the medium. Fluid culture media are used mainly as enrichment media, biochemical testing media and blood culture media. Examples are Tryptone broth, selenite F broth, Nutrient broth etc.

On the basis of utility purpose, the main types of culture media are:

Basal media : These are simple media which support the growth of microorganisms that do not have special nutritional requirements. Basal media are also known as **general purpose media**. These are often used in the preparation of enriched media, to maintain stock cultures of control strains of bacteria, and for subculturing pathogens from differential or selective media prior to performing biochemical and serological identification tests. Examples are, Nutrient agar, Nutrient broth.

Enriched media : These are media that are enriched with whole blood, lyzed blood, serum, extra peptones, special extracts, or vitamins to support the growth of pathogens that require additional nutrients or growth stimulants. Examples are Blood agar, loeffler serum medium, Tryptone soya agar etc.

Enrichment media : These are fluid media that increases the numbers of a pathogen by containing enrichments, and or substances that discourage the multiplication of unwanted bacteria. For example, selenite F broth is used as an enrichment medium for salmonellae in faeces or urine prior to subculturing on xylose lysine deoxycholate (XLD) agar or other enteric selective medium.

Selective media : These are media which contain substances that inhibit or slow down the growth of microorganisms other than the pathogens for which the media are intended. This type of media facilitate the isolation of particular species from a mixed inoculum. In recent years, antimicrobial agents have become increasingly used as selective agents in culture media. Examples are, XLD agar for *Salmonellae* and *Shigellae*, Butzler medium for *Campylobacter species*.

Differential media : These are media to which indicators, dyes or other substances are added to differentiate microorganisms. This type of media can also be known as **Indicator media** as this media incorporate some substance that is changed visibly as a result of the metabolic activities of particular organisms. For example, TCBS agar contains the indicator bromothymol blue which differentiates sucrose fermenting from non-sucrose fermenting *vibrio sps*. MacConkey agar contains the indicator neutral red which differentiates lactose fermenting from non-lactose fermenting organisms of enterobacteriaceae group. Blood agar can also be described as a differential medium when it differentiates haemolytic from non-haemolytic bacteria.

Transport media : These are mostly semisolid media that contain ingredients to prevent the overgrowth of commensals and ensure the survival of aerobic and anaerobic pathogens when specimens can not be cultured soon after collection. Their use is particularly important when transporting microbiological specimens from health centers to the district microbiology laboratory. Examples are, Cary-Blair medium for preserving enteric pathogens; Amies transport medium for ensuring the viability of gonococci and other Pathogens in specimens collected on swabs.

Table 5.1: Some of the Solid, Fluid, and Semisolid Culture Media are:

Medium	Main Uses
Blood agar	**Solid Culture Media** Enriched medium for culturing a wide range of pathogens and detecting haemolytic bacteria. Notes: Crystal violet blood agar is used as a selective medium for beta-haemolytic streptococci. Kanamycin and neomycin blood agar are used as selective media for anaerobes. Chocolate (heated blood) agar is used for the culture of *Haemophilus influenzae* and *Neisseria species*.
Butzler medium	Selective and enriched medium for isolating *Campylobacter species* from faeces.
Charcoal cephalexin blood agar (CCBA)	Selective and enriched medium for *Bordetella species*.
Deoxycholate citrate agar (DCA)	Selective and differential medium for enteric pathogens.
DNAse agar	Biochemical medium for identifying *Staphylococcus aureus*.
Dorset egg medium	Enriched medium for subculturing *Corynebacterium diphtheriae* to show volutin granules.
Kligler iron agar (KIA)	Differential medium for *Salmonella* and *Shigella species*.
Lactose egg yolk milk agar	Differential medium for identifying *Clostridium perfringens*.
Loeffler serum medium	Enriched medium for subculturing *Corynebacterium diphtheriae* to show volutin granules.

Lowenstein Jensen acid medium	For culturing *Mycobacterium tuberculosis*
MacConkey agar	For differentiating lactose fermenting from non-lactose fermenting bacteria.
Malt extract cycloheximide agar	Selective and enriched medium for ringworm fungi.
Mannitol salt agar	Differential and selective medium for *Staphylococcus aureus.*
Modified New York City (MNYC) medium	Selective and enriched medium for *Neisseria gonorrhoeae.*
Modified Tinsdale medium (MTM)	Selective and differential medium for *Corynebacterium diphtheriae.*
Motility indole urea (MIU) medium	Differential and motility medium for enterobacteria.
Nutrient agar	Basic medium for culturing bacteria that have no special nutritional requirements
Phenylalanine agar	Biochemical medium for differentiating *Proteus* and *Providencia* from other enterobacteria, and excluding *Yersinia enterocolitica.*
Sabouraud agar	For culturing fungi.
Tellurite blood agar	Selective medium for *Corynebacterium diphtheriae.*
Thiosulphate citrate bile salt sucrose(TCBS) agar	Selective and differential medium for *Vibrio cholerae* and other Vibrio species..
Tryptone soya medium (diphasic)	For culturing pathogens in blood.
Xylose lysine deoxycholate (XLD) agar	Selective and differential medium for Salmonella and Shigella species.

FLUID CULTURE MEDIA

Medium	Main Uses
Alkaline peptone water	Enrichment and transport medium for *Vibrio cholerae*
Christensen's urea broth	For detecting urease activity of *Proteus species*
Cooked meat medium (CMM)	For culturing anaerobes and maintaining control strains of bacteria
Glucose phosphate peptone water	For culturing enterobacteria in the Voges-Proskauer test
Koser citrate medium	For detecting citrate utilization in differentiating enterobacteria from the other bacteria
Litmus milk medium	For detecting litmus milk reduction in identifying enterococci and some *Clostridium species*
Nitrate broth	For detecting nitrate reduction in differentiating *Mycobacterium species* and Gram negative bacteria
Peptone water sugars	Carbohydrate testing medium for differentiating enteric pathogens
Selenite F Broth	Enrichment medium for *Salmonella species* in faeces and urine
Thioglycollate broth	For culturing anaerobes in blood
Todd-Hewitt broth	For culturing beta-haemolytic *streptococci* and other bacteria
Tryptone water	For detecting indole production, especially of *Escherichia coli.*

SEMISOLID CULTURE MEDIA

Amies transport medium	Transport medium for *Neisseria gonorrhoeae* and other pathogens.
Cary-Blair transport medium	Transport medium for faecal pathogens (*Salmonella, Shigella, Vibrio, Yersini* and *Campylobacter*)
Viral transport medium (VTM)	Transport medium for viruses.

11. Preparation of basic liquid media (broth) for the cultivation of bacteria.

Bacteria, in contrast to fungi, are often cultured in a liquid broth. Broth media are used mainly as enrichment media, biochemical testing media and blood culture media. The most common constituents of basic media used in routine Bacteriological laboratory are beef extract (a beef derivative which is a source of organic carbon, nitrogen, vitamins, inorganic salts and other accessory growth factors) and peptone (water soluble products obtained from the hydrolysis of animal or plant proteins). These may be modified in a variety of ways by supplementing with some specific chemicals or materials to provide a medium suitable for the cultivation or demonstration of a reaction for specific types or groups of bacteria.

Materials required:

1. Apparatus :

pH meter
Hot plate/Heater
Conical flask
Beaker
Measuring cylinder
Autoclave
Culture tubes
Cotton
Balance

2. Chemicals :

Nutrient broth Powder
1N HCl
1N NaOH
Distilled water

Procedure:

1. Weigh out the Nutrient broth powder for 1000 ml. media as instructed by manufacturer.

2. Put it in 500 ml. distilled water in a conical flask/beaker and heat it with agitation to dissolve.
3. Add more distilled water to make the volume 1 litre.
4. Adjust pH of the medium to 7.0 (as instructed) using a pH meter by adding either acid or alkali, as the case may be.
5. Pour 10 ml. of the broth per tube and apply the cotton plugs in each.
6. Autoclave all these tubes in an autoclave at 121°C 15 lbs pressure for 15 minutes.
7. Allow the autoclave to cool and remove the nutrient broth tubes.
8. Store the tubes at 4°C for further use.

Precautions:

1. The powder should be weighed accurately.
2. Cotton plugs are to be kept loose when autoclaving.

12. Preparation of basic solid media, agar slants and agar deep tubes for the cultivation of microorganisms.

The broth media are solidified by the addition of agar. Agar-agar (often called simply agar) is an inert polysaccharide extract obtained from a variety of red-purple seaweeds (rhodophyceae). Agar is a complex polysaccharide consisting of 3,6-anhydro-L galactose and D galacto-pyranose, free of nitrogen, produced from various red algae belonging to Geladium, Gracilaria and other genera. Agar is used to solidify culture media because of its high gelling strength and its setting temperature of 40-45°C and melting temperature of 90-95°C. The low gelling temperature allows heat sensitive nutrients such as whole blood to be added safely at 45-50°C. The solidified medium kept in a petridish provides an artificial environment suitable for the growth of microorganisms. While in the liquefied state, solid media can be added in test tubes, which are either allowed to cool and harden in a slanted position producing **agar slants** or allowed to harden in the upright position producing **agar deep tubes**.

On solid media, microorganisms multiply to form visible colonies. The colonial appearances (round, irregular, transparent or opaque, surface-smooth or rough, flat or raised central elevation or depression) and any changes in the surrounding medium (haemolytic reactions, pigment production, colour changes surrounding carbohydrate fermenting colonies and blackening due to H_2S production) help to identify bacteria. Preparation of Nutrient agar plate and slants will be discussed here.

Materials Required:

1. **Apparatus :**
   - Conical flasks
   - Glass tubes
   - Measuring cylinder
   - Cotton
   - Petridishes
   - Autoclave
   - Heater
2. **Chemicals :**

Nutrient agar powder (pH 7.0) having the composition

Peptone	5.0g
Beef extract	3.0 g
Agar	15.0 g
Distilled water	1000.0 ml

Procedure:

1. Take appropriate amount of nutrient agar powder and dissolve it in a conical flask by heating and make the volume to 1 litre.
2. Dispense 200 ml. each to five conical flasks. OR Dispense 8 ml each in screw-capped (or cotton pluged) tubes when slants or deep tubes are to be prepared.
3. Plug the flasks and test tubes containing medium.
4. Sterilize at 121°C, 15 lbs pressure, for 15 minutes in an autoclave.
5. Allow the tubes to cool in slanting position (for agar slants) and upright position (for agar deep tubes)
6. Allow the flasks to cool until the flasks can be held by hand.
7. Pour the medium into sterilized petridishes quickly under aseptic condition and allow the medium to solidify to produce agar plates.

Precautions:

1. Do not Pour medium over 2/3 capacity of the container used for autoclaving.
2. Cotton plugs should be kept loose when autoclaving.
3. Pour the medium into the petriplates so as to make the depth of medium 4 mm.

13. Pure Culture Techniques

In our environment (soil, air, water, food, sewage, body surfaces) microorganism exist in mixed populations. It is only in very rare situations that they occur as a single species. To be able to study the cultural, morphological, physiological and immunological characteristics of a species it is essential, first of all, that it be separated from the other organisms normally found in its habitat; in other words, we must have a pure culture of the microorganism. A culture that contains only one kind of microorganism is called a **pure culture**. A culture which contains more than one kind of microorganisms is called a **mixed culture**. Two kinds of operations i.e. **isolation** (the separation of a particular microorganism from the mixed populations that exist in nature) and **cultivation** (the growth of microorganisms in artificial environments such as culture media under laboratory conditions) come into play irrespective of the kind of microorganisms with which a microbiologist deals.

Pure cultures of microorganisms that form discrete colonies on solid media may be most simply obtained by one of the modifications of the plating method. Each viable organism gives rise, through growth to a colony from which sub-culture can be readily made. Several different methods of getting a pure culture from a mixed culture are available to us. The three most frequently used methods are (i) **Streak plate** (ii) **pour-plate** and (iii) **spread-plate method**. In all these methods, the techniques involve thinning out the organisms so that the individual species can be selected from the others.

Here, in this exercise, only the streak plate method is described. The pour plate and spread plate methods are described in **Section-VI** page number 92-96.

Streak Plate Method

The streak plate method is a most economical and practical method of obtaining discrete colonies and pure cultures. It was originally developed by two bacteriologists, Loeffler and Gaffkey in the laboratory of Robert Koch. It requires a certain amount of skill, however, which is forthcoming with experience. A properly executed streak plate will give as good an isolation as is desired for most work. In this method, a sterilized loop is dipped into a suitable diluted suspension of organisms which is then streaked on the surface of an already solidified agar plate to make a series of parallel, non-overlapping streaks. Streaking can be made in different ways.

Materials Required

Inoculating loop
Bunsen burner
Marker
Nutrient agar plates
24 hours mixed culture of *E.coli* and *S. aureus*.

Procedure:

1. Label all the plates, on the bottom, with the name of sample to be inoculated with the help of marker.
2. Hold the tube containing the sample in the left hand.
3. Sterilize the loop on flame holding in the right hand.
4. Remove the cotton wool plug from the sample tube and immediately flame the mouth of the tube.
5. Introduce the loop into the sample tube and withdraw one loopful of suspension.
6. Flame the mouth of the tube, replace the cotton wool plug and place the tube in the test tube rack.
7. Lift the Petri plate cover with the left hand and hold it at an angle of 60°.
8. Streak the plate by any one of the following methods.

(i) Quadrant streak method

a. Spread one loopful of organisms over a small area near the edge of the plate and streak it. This is area 1, apply the loop lightly to the medium to avoid digging into it.
b. Sterilize the loop by flaming and cool it for 5 seconds.
c. Make five or six streaks from Area 1 through Area 2. Stay near the edge of the plate.
d. Sterilize the loop again and allow it to cool.
e. Make six or seven streaks from Area 2 through Area 3.
f Sterilize the loop again and make as many streaks from Area 3 through Area 4 as possible.
g. Sterilize the loop again before putting it down.

(ii) Radiant Streak Method

a. Spread one loopful organisms over a small area near the edge of the plate. This is Area 1.
b. Sterilize the loop and allow it to cool for 5 seconds.
c. From the edge of Area 1 make seven or eight streaks to the opposite side of the plate.
d. Sterilize the loop again and cross-streak over the last group of streaks.
e. Flame the loop again before putting it down.

(iii) T streak Method

a. With the help of marker, draw a "T" on bottom of plate, dividing the plate into one half and two quarters.
b. Inoculate the half portion with one loopful of organisms in a continuous line from edge of the plate to the midline of the plate.
c. Sterilize the loop and cross-streak from Area 1 into Area 2 with one continuous streak, filling Area 2.
d. Sterilize the loop again and cross-streak from Area 2 to Area 3 with a single continuous streak.

(iv) Continuous Streak

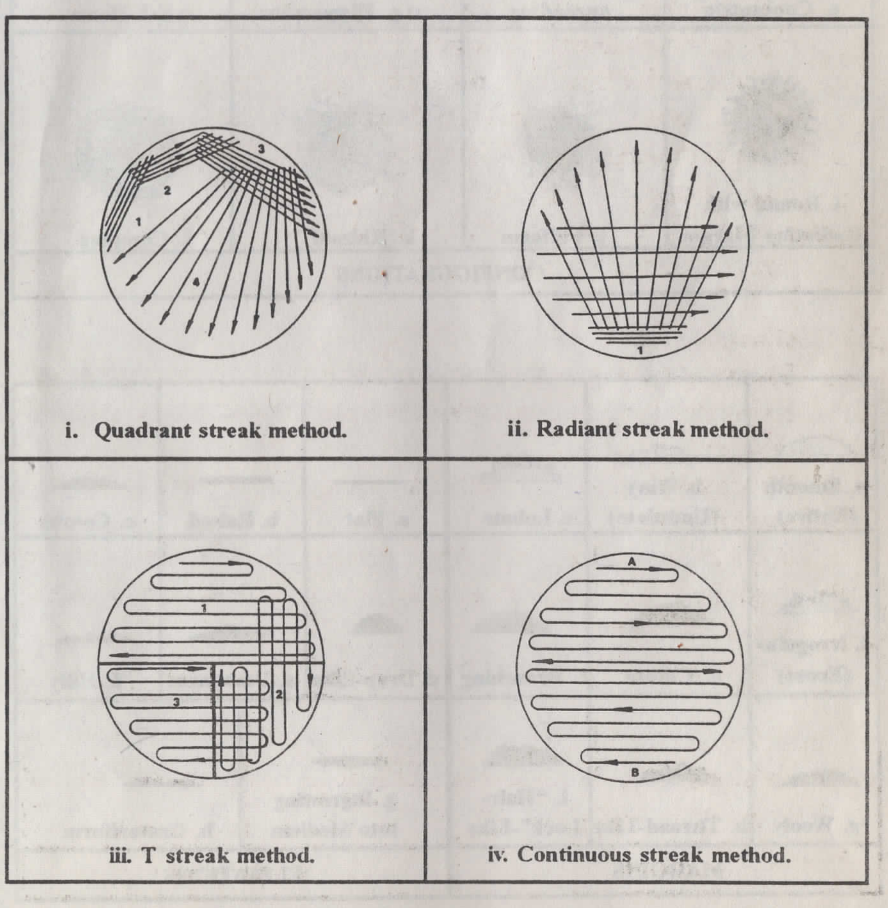
a. Starting at the edge of the plate (Area A) with a loopful of organisms, spread the organisms in a single continuous movement to the center of the plate.
b. Rotate the plate 180° so that the uninoculated portion of the plate is away from you.
c. Without flaming loop, and using the same face of the loop, continue streaking the other half of the plate by starting at Area B and working toward the center.
d. Flame your loop before setting it aside.

9. Incubate the plate in an inverted position at 37°C. for 24-48 hours. In this position, moisture which might form on the Petri plate cover will not be able to drop on the medium.

Observations

After incubation, examine each plate for the growth of organisms as colonies. Observe the colony characteristics of organism as:

S.No	Media	Colour	Shape	Size	Margin	Configuration	Elevation	Opacity



i. Quadrant streak method.
ii. Radiant streak method.
iii. T streak method.
iv. Continuous streak method.

Figure 5.1 : Techniques of Streaking.

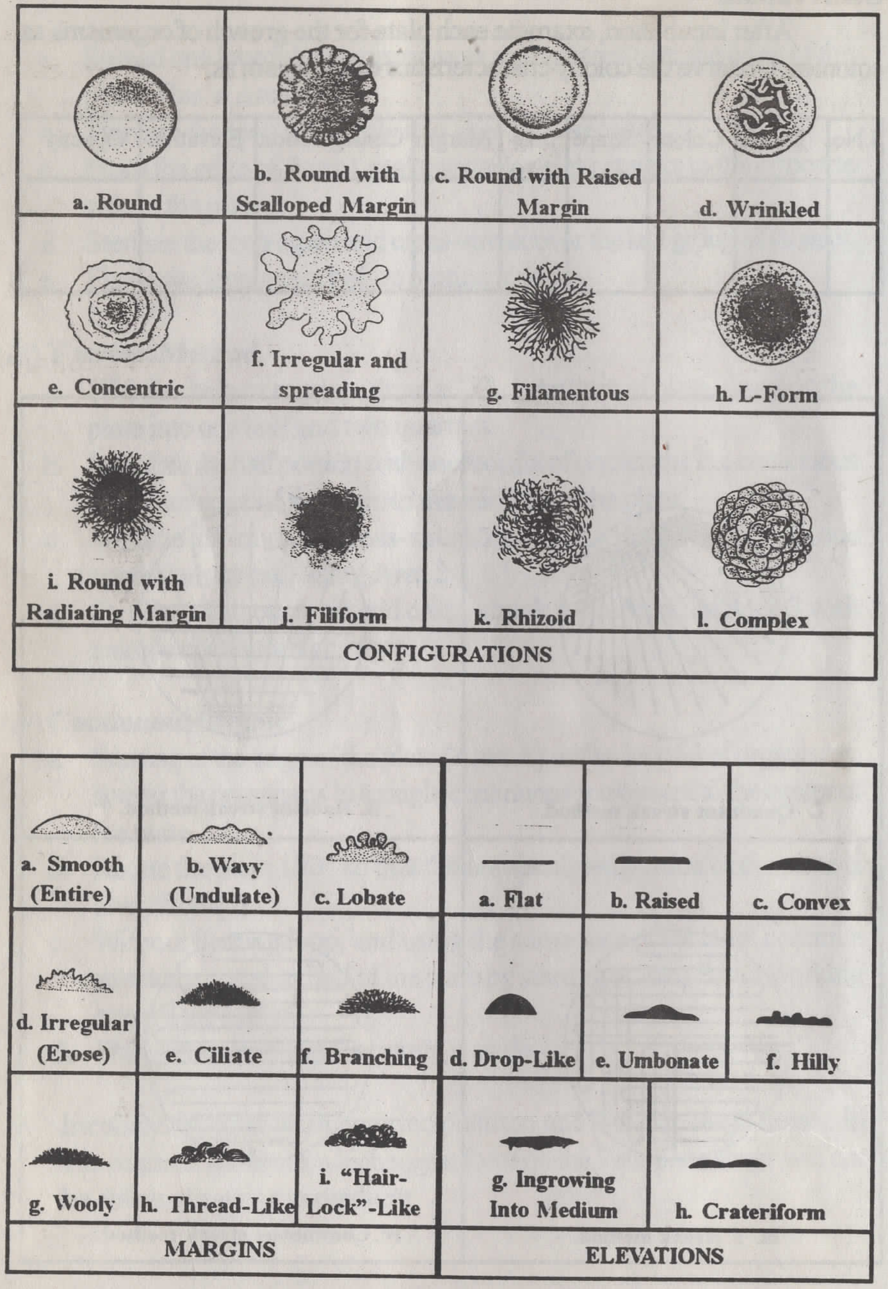

a. Round
b. Round with Scalloped Margin
c. Round with Raised Margin
d. Wrinkled
e. Concentric
f. Irregular and spreading
g. Filamentous
h. L-Form
i. Round with Radiating Margin
j. Filiform
k. Rhizoid
l. Complex
CONFIGURATIONS
a. Smooth (Entire)
b. Wavy (Undulate)
c. Lobate
d. Irregular (Erose)
e. Ciliate
f. Branching
g. Wooly
h. Thread-Like
i. "Hair-Lock"-Like
MARGINS
a. Flat
b. Raised
c. Convex
d. Drop-Like
e. Umbonate
f. Hilly
g. Ingrowing Into Medium
h. Crateriform
ELEVATIONS

Figure 5.2 : Colony Characteristics.

Result

A confluent growth will be seen where the initial streak was made, the growth is less dense away from the streak, and discrete colonies farthest away from the streak (i.e. end of the streak). Any colony not growing on the streak marks is regarded as a contaminant.

Precautions

1. Avoid pressing the loop or needle too firmly against the agar surface as this will damage it.
2. Inoculating loop should be cooled by touching the agar surface away from the set of streaks, before streaking of the inoculum.

Colony - distinct, compact masses of cells that are macroscopically visible.

14. Anaerobic Culture

Microorganisms vary in their need for, or tolerance of oxygen and accordingly, they are divided into several groups. **Aerobes** are capable of growth at full oxygen tensions (21% O_2 in air) and many can even tolerate elevated concentrations of oxygen (hydrobaric oxygen). **Microaerophiles**, by contrast, are aerobes that can use O_2 only when it is present at levels reduced from than in air, usually because of their limited capacity to respire or because they contain some oxygen sensitive molecule such as an oxygen-labile enzyme. Organisms that lack a respiratory system cannot use oxygen as a terminal electron acceptor. Such organisms are called **anaerobes**. There are two kinds of anaerobes: **aerotolerant anaerobes**, which can tolerate molecular oxygen and grow in its presence even though they cannot use it, and **obligate** or **strict anaerobes**, which are killed by molecular oxygen. The lethal effect on strict anaerobes is probably due to inability to detoxify some of the products of oxygen metabolism such as hydrogen peroxide, superoxide and hydroxyl radical formed. Many obligate anaerobes are rich in flavin eyzymes, which can react spontaneously with O_2 to yield these toxic products. By contrast, aerobes have enzymes that decompose toxic oxygen products, whereas anaerobes seem to lack all or some of these enzymes.

For anaerobic culture, the problem is to exclude oxygen, and because oxygen is ubiquitous in air, special methods are needed to culture anaerobic microorganisms. Obligate anaerobes vary in their sensitivity to oxygen; and a number of procedures are available for reducing the O_2 content of cultures, some simple and suitable mainly for less sensitive organisms, others more complex but necessary for growth of strict anaerobes. The isolation of anaerobic bacteria by plating methods poses special problems. Provided that the desired organisms are not rapidly killed by exposure to oxygen, plates may be prepared in the usual manner and then incubated in closed containers, from which the oxygen is removed either by chemical absorption or evacuation. [Bottles or tubes filled completely to the top with culture medium and provided with tightly fitting stoppers provide anoxic conditions for organisms not too sensitive to small amounts of oxygen]. For more oxygen sensitive anaerobes, a modification of the pour plate method known as the **dilution shake culture**, is preferred. For strict anaerobes, techniques as **roll tube** and **anaerobic globe box** are employed.

Methods for cultivation of anaerobes

(1) Evacuation and replacement of oxygen atmosphere in sealed jars.

a. Brewer anaerobic culture plate

b. The Gas Pak system

c. Vacuum and Gas Displacement Method

d. Anaerobic chamber

e. The candle method.

(2). Specialized techniques where evacuated sealed jars are not used.

A. Solid Medium

- a. Shake culture method
- b. Pyrogallic acid method
- c. Roll tube method

B. Liquid medium

- a. Paraffin plug method

Plate Methods

There are several techniques of growing anaerobic bacteria on an agar plate. The most commonly used methods are Brewer's anaerobic plate, Gas Pak Jar, vacuum Gas displacement method.

i. Brewer's Anaerobic Culture Plate

In 1942, J.H. Brewer, introduced a especially designed petridish cover to be used with an anaerobic medium for the surface cultivation of anaerobes. It is a relatively simple device and gives satisfactory results. The cover is provided with a circular ridge that rests on the medium, providing very rare space above the medium. Anaerobic condition in the space results from the presence of reducing agents in the medium.

After streaking on the agar surface, the conventional petri dish cover is replaced by the sterile Brewer cover. The medium should be deep enough so that the Brewer cover does not rest on the edge of the bottom dish. The weight of the cover will cause the cover ridge to settle somewhat into the medium.

When incubating the plates, avoid stacking more than 3-4 plates on top of each other. The pressure exerted on the bottom plate becomes excessive when too many plates are stacked.

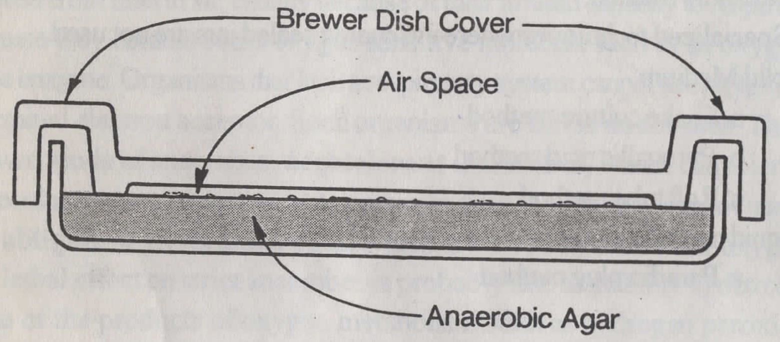

Brewer Dish Cover
Air Space
Anaerobic Agar

Figure 5.3 : Brewer's Petri Dish Method.

ii. The Gas Pak Jar

The Gas Pak Anaerobic system is composed of the Gas Pak generator envelope and the anaerobic indicator strip kept inside an anaerobic jar. Media plates are inoculated and kept inside the jar. Water is added to the Gas Pak generator envelope, causing the evolution of H_2 and CO_2. The H_2 reacts with O_2 on the surface of palladium catalyst, forming H_2O and establishing anaerobic environment carbon dioxide is also produced which enhances the growth of fastidious bacteria which sometimes fail to grow, or grow only poorly in its absence. To make sure that anaerobic conditions actually exist in the jar, an indicator strip saturated with methylene blue is kept inside the jar. The pad changes from blue to colourless in absence of oxygen. While using anaerobic jar, following steps should be taken well into consideration.

1. Peel apart the foil at one end of a Gas Pak indicator strip and pull it halfway down. The indicator will turn blue on exposure to the air. Place the indicator in the jar as such.
2. Cut the corner of each of three Gas Pak gas generator envelopes with a pair of scissors. Place them in jar in upright position.
3. Pipette 10 ml of water into the open corner of each envelope.
4. Place the double lids on to the jar in positions tighten the knobs before keeping the jar in the incubator.

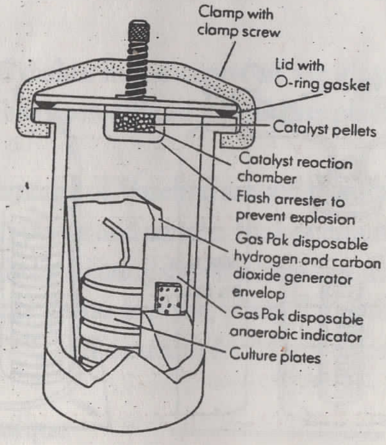

Clamp with clamp screw
Lid with O-ring gasket
Catalyst pellets
Catalyst reaction chamber
Flash arrester to prevent explosion
Gas Pak disposable hydrogen and carbon dioxide generator envelop
Gas Pak disposable anaerobic indicator
Culture plates

Figure 5.4 Gas Pak System.

In Brewer's jar, hydrogen or a mixture of gases (95% N_2 and 5% CO_2) is introduced into the apparatus after it is sealed. Heat activation of the platinum catalyst in the jar lid results in binding of residual oxygen with hydrogen with the formation of water, thus creating anaerobic environment.

iii. Vacuum and Gas Displacement Method

Ideal atmospheric conditions for strict anaerobes can be achieved by displacing the air in a closed container with a mixture of nitrogen and carbon dioxide. A vacuum pump is used to withdraw the air form the chamber containing the culture plates to be incubated. A mixture of the two gases is then allowed to fill the chamber. The CO_2 serves the same purpose as in Gas Pak System. Many bacteria have difficulty starting growth in absence of CO_2. Once growth has been established, however, increased CO_2 concentration is usually unnecessary. In this method, a tank of gas (95% nitrogen and 5% CO_2) is used.

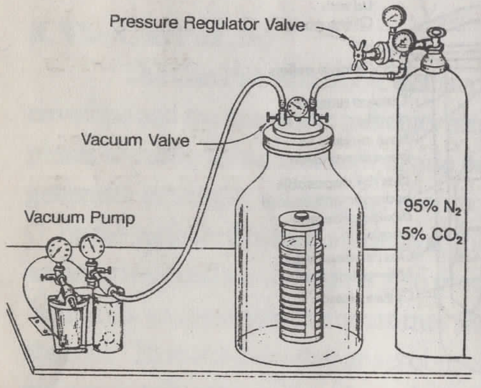


Figure 5.5 : Vacuum and Gas Displacement Method.

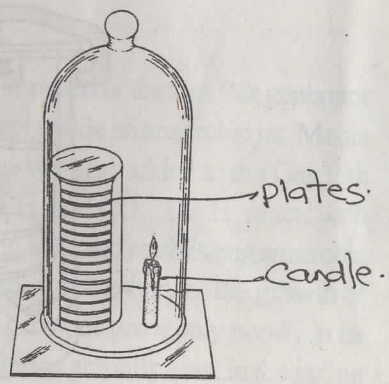


Figure 5.6 : Candle Method.

iv. Anaerobic Chamber

This refers to anaerobic globe box which is a sealed chamber in which objects are manipulated with hands inserted into rubber gloves that are attached to the front of the unit; a transparent panel allows the manipulation to be viewed from the outside. The chamber has an atmosphere of H_2, CO_2 and N_2. Culture media are placed within the chamber by means of an air lock, which can be evacuated and refilled with N_2. From the air lock, media are placed within the main chamber. Any O_2 in the media is slowly removed by reaction with H_2, forming water. This reaction is aided by a palladium catalyst. After being rendered O_2-free, the media are inoculated within the chamber (by means of the globe ports) and incubated (also within the incubator).

v. The Candle Method

Although this method is an old one, it is still used for culturing some bacteria such as *Neisseria gonorrhoeae*. For many organisms, however, it is considered too toxic because of the amount of carbon monoxide produced by the burning candle. When a lighted candle is placed in a container that is tightly sealed, the O_2 will be used up, CO_2 will be produced, and the candle will quit burning after the O_2 has been exhausted.

Procedure :

1. Place the petri dishes in the center of the glass plate and light the candle which has been affixed to the glass plate with melted candle wax.
2. Apply a little amount of Vaseline around bottom of bell jar and place it over the petri plates and candle. Make certain that the jar-to-glass plate seal it tight by rotating the jar a little against the glass plate.
3. After the candle flame expires, place the entire assembly into an incubator at 37°C and incubate for 24 to 48 hours.

Shake Culture Technique

A tube of molten, cooled (45°C) agar medium is inoculated with the test organism. The tube is shaken, cooled rapidly and incubated. Upon incubation, different oxygen requirements of organisms may be manifested as follows.

a. Strict anaerobes grow in the deeper portions of the culture.
b. Facultative anaerobes are found throughout the preparation.
c. Microaerophiles grow near the surface of the medium
d. Aerobes grow at the surface of the medium
e. Aero tolerant anaerobes grow throughout the medium.

A redox dye as resazurine may be added to the medium because the dye changes colour in the presence of oxygen and thereby indicates the degree of penetration of oxygen into the medium. It is also possible to add a reducing agent that reacts with oxygen and reduces it to H_2O. A good example is thioglycolate which is added to a medium called thioglycolate broth. After thioglycolate reacts with O_2 throughout the tube, oxygen can penetrate only near the top of the tube where the medium contacts air.

Shake culture technique may be slightly modified by preparing series of dilutions too. The technique is then known as dilution shake culture.

A layer of sterile petroleum jelly and paraffin may be added on the surface of medium before incubation to prevent access of air to the agar column. In shake culture, the colonies develop deep in the agar column and thus are not easily accessible for transfer. To make a transfer, the seal (if used) is removed with a sterile needle, and the agar column is extruded from the tube into a sterile petridish by gently blowing a stream of gas through a capillary pipette inserted between the tube wall and the agar. The column is sectioned into discs with a sterile knife to permit examination and transfer of colonies.

Procedure

1. Take a nutrient agar deep and label it.
2. Melt the nutrient agar in the test tube
3. Allow it to cool to 45°C
4. Inoculate the test organism into molten, cooled medium aseptically.
5. Shake the tube by gently striking it with your fingers.
6. Cool the tube rapidly under running cold water to solidify the medium
7. Incubate the tube at 37°C for 24-48 hours.
8. Repeat the same process for other test organisms also.

Observation and Results: Examine the tubes and observe for the regions where the growth occurred.

Wright's Tube Method

This method is also called **pyrogallic acid sodium hydroxide method**. To grow anaerobes on a nutrient agar slant, one can create anaerobic conditions within the tube by using pyrogallol and sodium hydroxide. When activated with sodium hydroxide, pyrogallol acts as a powerful reducing agent to remove the oxygen within the tube. The outer plug of the tube should significantly be of rubber or cork and be air tight Inoculation of a control tube enables us to determine the relative oxygen needs of different organisms and the effectiveness of a Wright's tube.

Procedure

1. Take a nutrient agar slant and label it.
2. Aseptically, inoculate slant with the test organism by streaking the agar surface.
3. Replace the cotton plug.
4. Ignite the plug by passing it through the bunsen flame. Alternatively, cut off the cotton plug outside the tube with a pair of scissors.
5. Using a glass rod, push the cotton plug into the tube until it nearly touches the slant.
6. Add pyrogallol (pyrogallic acid crystals) to fill the space between the cotton and top of the tube.
7. Add few ml of sodium hydroxide (4%) on the top of pyrogallol.
8. Apply a rubber stopper immediately and force it down to the tube.
9. Invert the tube quickly to prevent seepage of the solution down onto the agar slant.
10. Incubate the tube in inverted position at 37°C for 24-48 hours.
11. Repeat the same procedure for control i.e. strict anaerobe as *Clostridium sporogens*.

Observation and Results : Examine the incubated slants for the presence or absence of bacterial growth. *Clostridium sporogenes* growth will occur on the nutrient agar slant.

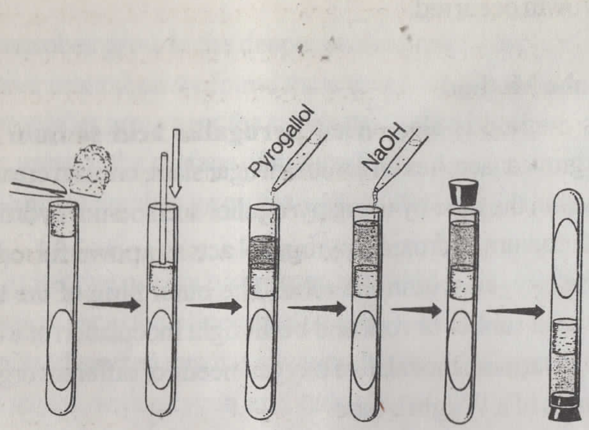


Figure 5.7. Wright's Tube Method.

Roll Tube Technique

The role tube procedure, developed by R.E. Hungate, is used to obtain pure cultures of strict anaerobes by distributing cells in a thin layer of agar on the walls of a test tube, where they develop into isolated colonies. The test tube contains few ml of medium that has been reduced chemically to remove dissolved oxygen and is tightly stopperred with a butyl rubber bung (small but lethal quantities of oxygen diffuse through ordinary rubber). The molten agar is inoculated with appropriate dilution of organism inserting through the rubber stopper with a sterile syringe.

The tubes are then laid on their sides in ice and rolled until the agar solidifies in a thin layer on the wall of the tube. After the period of incubation when colonies become visible, the bung is removed and isolated colonies picked from the agar with a needle or capillary tube. Whenever a tube is unstoppered, entry of air is prevented by continuously passing a stream of O_2 free gas (normally CO_2 and N_2) into the tube. To ensure that some entry of air has not inadvertently occurred, it is usual to include in the medium the redox dye, resazurine.

Paraffin Plug Method

This method is usually employed to create anaerobic environment in broth culture. After the inoculation of organism into suitable liquid or semisolid medium, immediately a layer of sterile paraffin oil is used to cover the medium. The culture tube is then stoppered (cotton plug) and incubated. Paraffin plug method is widely applicable in studying the metabolic activities of the organisms. For example, during oxidation fermentation test of an organism, duplicate media tubes are used. One of them is covered with a layer of paraffin during incubation for the anaerobic metabolism (e.g. fermentation) of the organism if present.

Pre-reduced media preparation

The culture media is boiled for several minutes to drive off most of the dissolved oxygen. A reducing agent. eg. cysteine, H_2S, is added to further lower the oxygen content. Oxygen free N_2 is bubbled through the medium to keep it anaerobic. The medium is then dispensed into tubes, which are being flushed with O_2-free N_2, stoppered tightly and sterilized by autoclaving. Such tubes can be stored for many months before being used. During inoculation, the tubes are continuously flushed with oxygen free CO_2 by means of a canula, restoppered and incubated. Inoculation is best carried out by using Pasteur's pipette.

Section - VI

Determination of Number of Microorganisms

Quantitative measurements are usually done to follow the course of growth. Since the exponential growth phase is balanced phase, any property of the biomass can be measured to determine growth rate. As for convenience, the properties measured are usually cell mass and cell number. The only direct way to measure cell mass is to determine the dry weight of cell material in a fixed volume of culture by removing the cells from the medium, drying them and then weighing them. Such determinations are time consuming and relatively insensitive. With an ordinary equipment it is difficult to weight with accuracy less than 1 mg, yet this dry weight may represent approximately as many as five billion bacteria. The method of choice for measuring cell mass of unicellular organisms is an optical method; the determination of amount of light scattered by a suspension of cells. This technique is based on the fact that small particles scatter light proportionally, within certain limits, to their concentration. Such measurements are usually made in a photometer or spectrophotometer. More sensitive instruments for measuring scattering are called nephelometers.

The number of bacteria in a suspension can be determined microscopically by counting the individual cells in an accurately determined small volume. Such counting is usually done with special microscope slides called counting chambers. Such a direct count is known as the **total cell count** that includes both viable & nonviable cells. The principal limitation of direct microscope count is the relatively high concentrations of cells that must be present in the suspension. The high magnification required for seeing the bacteria limits the volume of liquid that can be examined carefully with the microscope, yet, a significant number of cells per milliliter can be counted with any degree of accuracy. An electronic instrument, called counter, can also be used for the direct enumeration of cells in a suspension.

The enumeration of bacteria can also be made by plate count, because single viable cells separated from one another in space by dispersion on or in an agar medium give rise through growth to separate, macroscopically visible colonies. Hence, by preparing appropriate dilutions and using them to seed an appropriate medium, one can ascertain the number of viable cells in initial population by counting the number of colonies that develop after incubation and multiplying this figure by the dilution factor. This method of enumeration is called **viable count**, which is by far the most sensitive method of estimation bacterial number, since even a single viable cell in a suspension can be detected. A combination of total cell count and viable count can be used to determine the fraction of viable cells in a population. Alternatively, the viable fraction can be determined directly, by a technique that combines microscopic examination and growth.

Sometimes because of growth patterns (e.g. filaments, clumps) or of complexity of the medium, it is impractical to measure cell mass and numbers. In these cases, growth can be measured by determining the amount of particular cell constituent (eg. Protein, DNA, RNA or, ATP) in the medium. Such measurements are often the most practical way to determine microbial mass and growth in a natural environment.

Each of the methods has its particular advantages and limitations and no one method can be recommended universally. The best procedure can be selected only after these procedures are considered in relation to the problem at hand.

Microscopic count-(no. of cells /ml) – enumeration of bacteria in cultures, vaccines

Electronic enumeration-(no. of cells/ml) enumeration of bacteria in cultures, vaccines.

Plate count-(cfu/ml) – enumeration of bacteria in milk, water, food, soil, cultures

Membrane filter-enumerationof bacteria in milk, water, food, soil.

Turbidimetric measurement-(optical density/absorbance) – microbiological assay, estimation of cell in broth, cultures, suspensions.

Nitrogen determination-(mg N_2/ ml) – measurement of cell from heavy suspensions to be used for research in metabolism.

Dry weight determination-(mg dry wt of cells/ml)

Measurements of biochemical activity-(e.g. acid production by cultures) (milli equivalents of acid/ml or per culture) – microbiological assays.

Direct count-e.g.; i) Breed smear (for dried sample)

ii) Neubauer's chamber (for liquid samples)
iii) Electronic cell counter (for liquid samples)

Indirect counts

- Determining cell mass (dry wt)
- Determining cell constituents (DNA, RNA, protein)
- Oxygen uptake, Co_2 production
- Turbidimetric Measurements (spectro photometric or colorimetric analysis)
- Membrane filter count (Count living cell only)
- Serial dilution –agar plate (Plate count) method. (Count living cell only).

15. Breed Count Method

Breed count technique, designed by Robert Breed, American bacteriologist is both viable and non viable count method in which number of bacteria or yeast or algae or any organism in a sample of broth culture, milk, beer, fruit juices, etc may be determined by direct microscopic observation.

In this method of direct count, a known volume of sample (0.01ml) is uniformly spread over a 1 cm^2 area of a slide. The resulting film is dried and stained. The average number of organism per microscopic field is counted. The diameter of microscopic field is determined by micrometry, which is used for calculating the area of microscopic field. Then, the number of bacteria in the sample is calculated by using the formula

$N (T. C) = n \times F$

Where N (T.C.) = total number of organism/ ml

n = average number of organism in one microscope field

$$F = \frac{\text{Area of smear}}{\text{Area of microscopic field (A)}} \times 100$$

$A = \pi r^2$ (r-radius of microscopic field)

This method always gives high number of organisms than plate count or any other methods as this includes living as well as dead and inactive organisms. In other words, it does not distinguish between living and dead cells and this is a drawback of direct count.

Other limitations of direct count are small cells are difficult to see under microscope and some cells are probably missed – precision is difficult to achieve. A phase contrast microscope is required when the sample is not stained. It is not suitable for cell suspensions of low density. This technique is widely used in creameries, health departments and in daily estimation of quality of milk, diagnosing the types of contamination and growth in pasteurized milk products.

In addition to being more faster than SPC, the direct microscopic count has two other distinct advantages. First of all, it will reveal the presence of bacteria that do not form colonies on agar plate at 37°C (thermophiles, psychrophiles, dead bacteria). Second advantage is related to milk testing. The presence of leucocytes and pus forming streptococci on a slide will be evidence that the animal that produced the milk has an udder infection (mastitis). For milk and milk products, the fat content of the sample should be removed before staining by xylol and alcohol treatment of dried smear on the slide.

The number of fields to be counted is determined on the basis of no. of organism seen under one microscopic field as given in Wilson' chart .

Average no. of organism	No. of fields to be counted
0-3	64
4-6	32
7-12	16
13-25	8
26-50	4
51-100	2
>100	1

Observation and calculation

Suppose

Diameter of 1 microscopic field

=13 divs of stage micrometer
=(13X0.01) mm = 0.13 mm
(1 div of stage micrometer = 0.01 mm)
Therefore,

$$\text{Radius } (r) = \frac{\text{Diameter}}{2} = \frac{0.13}{2} = 0.06 \text{ mm}$$

And Area (A) $\pi r^2 = 0.01 \text{mm}^2$
Area of smear (sample spread, dried and fixed)
$= 1 \text{cm}^2 = 100 \text{mm}^2$
Therefore, No. of fields possible under lens = 100/0.01 = 10,000 fields.

This number of fields is for 0.01 ml sample. However final count is expressed in terms of no. of organism /ml of sample. Thus, this factor is to be multiplied by 100.

i.e 10,000X100 = 1,000,000 is the microscopic factor. Suppose, 25 fields yielded an average of 0.2 organism per microscopic field
Then final count would be
= 0.2 X 1,000,000
= 2000,000 organisms/ml of sample = $2\text{x}10^6$/ml.

Materials Required:

1. Breed pipette/capillary pipette
2. Glass slide (1 cm^2 engraved)
3. Methylene blue dye solution
4. Immersion oil
5. Microscope
6. Stage micrometer
7. Sample

Procedure

I. Determination of area of 1 microscopic field:

a. Put the stage micrometer on the stage of microscope and get sharp focus of it under oil immersion objective.
b. Count the no. of divisions of stage micrometer covered by one microscopic field.
c. Determine the radius and area of 1 microscopic field.

II. Enumeration of microorganisms.

a. Take a clean 1 cm^2 engraved glass slide (Make 1 cm^2 mark on the slide).
b. Put 0.01 ml of sample (using pipette or loop) and make uniform smear within the given marked area.
c. Air dry and heat fix (dry in incubator for heat fixation without cell shrinkage).
d. Perform simple staining with methylene blue.
e. Observe the smear under oil immersion.
f. Get concurrent readings on the basis of Wilson's chart.
g. Calculate the no. of organisms per ml of sample.

Precautions

1. Determine the area of microscopic field with respect to the objective lens you are using for cell count.
2. Do not over stain the slide.

16. Measurement of Cell Concentration by Using a Counting Chamber

The haemocytometer (so named because it was originally devised for counting blood cells) is used for counting the organism in liquid medium. It is a special microscopic slide with a counting chamber 0.1 mm deep so that the volume over a 1 sq. mm is 0.1 mm^3. The counting chamber has a total of nine squares, each of 1 mm x 1 mm engraved over it but only one square field is visible under 100 x magnification. A 1 mm^2 in the centre is divided into 25 medium sized squares (0.2 mm x 0.2 mm each), each of which is further subdivided into 16 small squares (0.05 mm x 0.05 mm each) thus a total of 400 sub-squares in 1 mm^2. Each medium sized square is separated by triple lines, the middle one acts as the boundary. The 1 mm^2 squares at four corners, each is subdivided into 16 sub squares (0.2 mm x 0.2 mm each) (thus a total of 16 sub squares in 1 mm^2). The volume of each large square at corners and at centre is (1 x 1 x 0.1) mm^3 or 0.1 mm^3 = (1/10 x 1/10 x 1/100) and or

1/1000 cm^3 or 10^{-4} cm^3. The volume of each square with 16 sub squares in the central square is 0.2x0.2x0.1 = 0.004 mm^3.

Counting of bacteria is carried out by the use of a **Neubauer's or Petroff Hausser counting chamber**, which is only 0.02 mm deep. The number of cells per unit area can be counted under the microscope, giving a measure of the number of cells per small chamber volume. Converting this value to the number of cells per ml of suspension is easily done by multiplying by a conversion factor based on the volume of chamber used. The total no. of cells per mm^3 is given by

N = n/v X D.f

Where N – total no. of cells /mm^3

n – total no. of cells counted in one chamber.

v- Volume of chamber used

D.f – Dilution factor = $\frac{1}{\text{Dilution}}$

Materials Required:

1. Counting chamber slide
2. Cover slip
3. Pipette / dropper
4. Microscope
5. Immersion oil.
6. Sample suspension

Procedure

1. Take a clean counting chamber slide and get sharp focus of the engraved chambers.
2. Put a cover slip on the chamber.
3. Apply the tip of dropper (pipettes) between the cover slip and platform of slide.
4. Allow some sample to flow in the narrow space (applying constant gentle pressure on cover slip)
5. Count the number of cells in all chambers.

[Put a drop of sample suspension on the engraved grid and put the cover slip over it carefully so that no air bubble enters between the slide and cover slip. Put a drop of stain into the suspension for better visualization of organisms.]

6. Use the corner squares for counting larger cells as fungal spores, yeast cells, WBC and use the central square for counting smaller cells as RBC and to have a higher total count of 200-250.

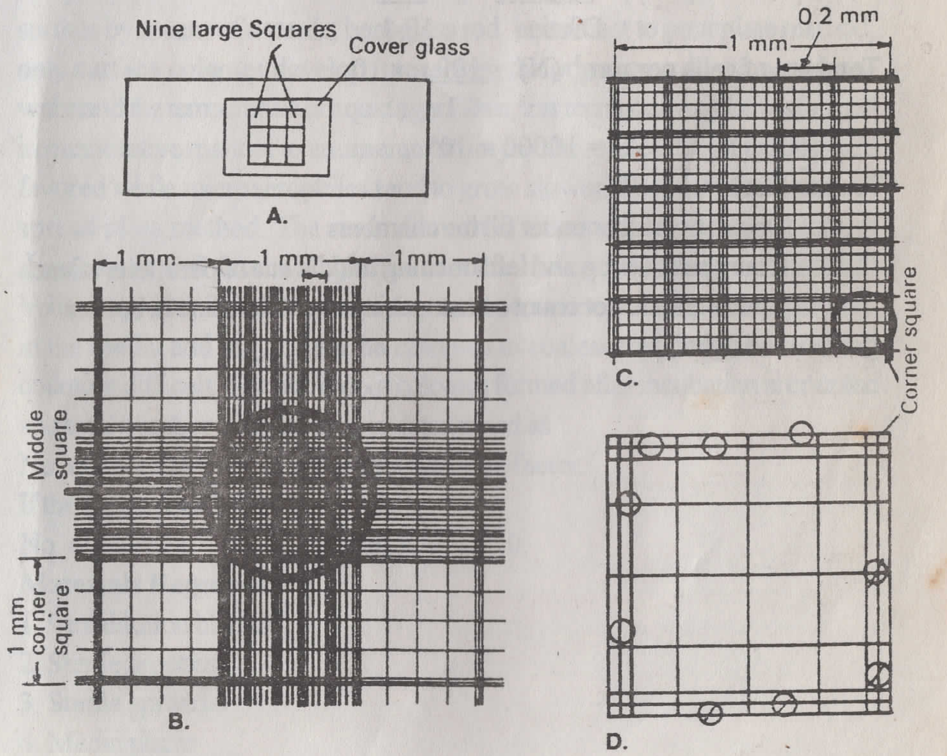


Figure 6.1 : (A) Haemocytometer (B) The Circle Indicates the Approx. Area Cover at 100 X Microcope (C) A Single Large (Middle) Square Enlarged (D) Corner Square Enlarged.

Observation and Results

Suppose,

Volume of each corner square of haemocytometer = $(1 \times 1 \times 0.1)\ mm^3$

$= 0.1 mm^3$

Total no of cells counted (n) = A+B+C+D=100

A- no of cells in first row

B- no of cells in second row

C- no of cells in third row

D- no of cells in fourth row

$$\text{Dilution factor (D.f)} = \frac{1}{\text{Dilution}} = \frac{1}{10\text{-}1} = 10$$

$$\text{Total no. of cells per } mm^3 \text{ (N)} = \frac{100}{0.1} \times 10$$

$$= 10000 = 10^4$$

Precautions

1. Do not overfill or under fill the chambers
2. Count cells on top and left touching middle line of perimeter of each square and do not count cells touching the middle line at bottom and right.

Plate Count (viable cell count) Method

17. Isolation and Enumeration of organisms by Spread Plate Technique

Spread plate technique aids in the isolation as well as enumeration of the organisms in a mixed culture. The mixed culture is first diluted to provide only a few cells per millilitre before being used to inoculate media. Since the number of bacteria in the specimen is not known beforehand, a series of dilutions are made so that at least one of the dilution will contain a suitably sparse concentration of cells. Usually dilution is done in series of tubes containing a sterile liquid, water or physiological saline. Measured amount of diluted sample is placed on to the surface of agar plate and spread evenly over the surface by means of a sterile, bent glass rod. In contrast to pour plate method, only surface colonies develop; moreover, the organisms do not require to withstand the temperature of liquid agar. Thus, this technique may be performed in quantitative manner to enumerate strict psychrophiles. Strict aerobes are favored while microaerophiles tend to grow slower. These are the merits of spread plate method. The main demerit of this method is the problem of spreaders as well as crowding of colonies that makes the enumeration difficult. Volumes greater than 0.1 ml are rarely used because excess liquid is not soaked in the media and may cause the colonies to coalesce as they form making counting difficult. The number of colonies formed after incubation is counted and total number of organisms is determined as

No. of bacteria = no. of colonies x Dilution factor.

If the inoculum size is 0.1ml,

No. of bacteria = no. of colonies x D.f. x 10.

Materials Required:

1. 9 ml dilution blanks
2. Sterile pipettes
3. Sterile spreader
4. Media plates
5. Bunsen burner
6. Sample

Procedure

I. Serial dilution of sample

1. Label the dilution blanks as 10^{-1}, 10^{-2} ....10^{-8}.
2. Prepare initial dilution by adding 1 ml or 1 gm of sample into 9ml dilution blank (10^{-1}) thus diluting the original sample. 10 times (1/1+9 = 1/10 and is written as 1:10 or 10^{-1})
3. Mix the content by rolling the tube back and forth between your hands to obtain uniform distribution of cells.
4. From the first dilution, transfer 1ml of the suspension while in motion, to the dilution blank 10^{-2} with a sterile pipette diluting specimen to 100 times (1/10 x 1/10 = 1/100 or 10^{-2})
5. Repeat this procedure till original sample has been diluted 100000000 times (10^{-8} dilution)

II. Inoculation

1. From the appropriate dilutions, transfer 0.1ml of sample into separate, dried nutrient agar plates.
2. Spread the inoculated sample using sterile spreaders throughout the media plate.
3. Incubate at 37°C for 24 hours.

III. Observation

S.N.	Sample	Dilution	No. of Organisms	Total no. of organisms (Dilution X no of organism)	Average no. of organisms

IV. Enumeration and Calculation

- Count the no. of colonies in the plates.
  [The usual practice, which is most valid statistically, is to count only on plates that have between 30-300 colonies.]
- Calculate no. of bacteria per ml of original sample as follows.

$$\text{No. of organisms per ml of sample} = \frac{\text{No. of colonies}}{\text{inoculum size x dilution}}$$

Example

If an average of 5×10^5 organism has been obtained using 0.1 ml inoculum size,

$$\text{No. of organism/ml} = \frac{5x10^5}{0.1} = 5 \times 10^6$$

Precautions

1. Sample should be evenly distributed and completely absorbed before incubation.
2. The moisture content of media surface should be dried off.

18. Isolation and Enumeration of Organisms by Miles and Mishra Method

This method of surface viable count consist of placing drops (0.02 ml) of bacterial sample to be enumerated on the surface of solid agar plate and counting the colonies which develop upon incubation of the plates. This method is useful when the bacteria are best grown on surface culture (e.g. when the presence of obligate aerobes is suspected) or when an opaque medium is employed. Surface counts are possible when the microbial population in a sample is likely to include bacteria which are killed by the brief exposure to 45-50°C which occurs while using molten agar for pour plate technique. The important precaution to be taken during this enumeration technique is that the dilutions poured from a height of 2.5cm using calibrated dropping pipette should be allowed to be absorbed completely before incubation. Miles and Mishra is the reliable technique for Surface Viable Count (SVC) and also may be called modified technique to pour plate technique for total viable count.

After counting the no. of colonies, the total number of organisms is determined as

$$\text{No. of organisms} = \frac{\textit{No.}\text{ of colonies x Dilution factor}}{\text{Inoculum size.}}$$

$$= \frac{\textit{No.}\text{ of colonies}}{\text{Dilution x Inoculum size}}$$

Materials Required:

Calibrated pipette (sterile)
Dilution blanks
Nutrient agar plates,
Sample,

Procedure

- Prepare serial dilutions as in spread plate technique.
- Drop 0.02 ml of selected dilutions from 2.5 cm height into NA plates.
- Incubate at 37°C for 24 hours.
- Count the no. of colonies and calculate the number of organisms per ml. of given sample

$$\text{No. of organisms per ml} = \frac{\text{No. of colonies x D.f}}{0.02}$$

Precautions

1. The moisture content of the media surface should be dried off.
2. The sample should be dropped with care from low height (not < 2.5cm) and should be absorbed completely before incubation.

19. Isolation and Enumeration of Organisms by Pour Plate Technique

The plate count or serial dilution – agar-plating technique is one of the most routinely used procedure for the enumeration of viable cells. This method is based on the principle that when material containing bacteria are cultured, every viable bacterium develops into a visible colony on a nutrient agar medium. The number of colonies, therefore, is the same as the number of organisms contained in the sample. In this method, a measured amount of sample is mixed with a large volume of sterile water or saline called diluent or diluent blank. Dilutions are usually made in multiples of ten.

A single dilution is calculated as :

$$\text{Dilution} = \frac{\textit{vol.}\text{ of sample}}{\text{total vol. of sample and diluent}}$$

Serial dilutions are then prepared by transferring a known volume of the dilution to second dilution blank and so on. Once diluted, the specific volume of diluted samples from various dilutions is added to sterile petriplates to which molten and cooled (45-50°C) suitable agar medium is added. The sample and medium are homogeneously mixed before solidification and then incubated. The number of colonies are counted, averaged and total number of organisms per ml of given sample is calculated as:

$$\text{No. of organisms per ml.} = \frac{\text{No. of colonies}}{\text{Inoculum size x Dilution}}$$

Advantages

- Somewhat less skill is required to get good isolation.
- Isolation as well as enumeration can be done.

Disadvantages

- Some organisms are trapped beneath the surface of medium when it gels and therefore both surface and subsurface colonies develop.
- The organisms being isolated must be able to withstand the temporary exposure to 45°C temperature of liquid agar medium, unsuitable for isolating psychrophiles. However, some micro-aerophiles and some spores may be benefited.

Materials Required:

1. Dilution blanks
2. Sterile pipettes
3. Sterile petriplates
4. Molten nutrient agar medium.
5. Sample

Procedure

i. Serial Dilution

1. Take 7 test tubes each with 9ml diluent and label them (10^{-1}, 10^{-2},.... 10^{-7})
2. Pipette out 1ml of given sample into first dilution blank, mix well
3. From 1st test tube (10^{-1} dilution), again transfer 1ml to 2nd test tube.

4. Repeat the process up to the last test tube such that 10^{-1}, 10^{-2} … 10^{-7} dilutions of given sample are prepared.

ii. Agar Plating

1. Transfer 1ml of aliquots from selected dilutions (say 10^{-3}, 10^{-5}, 10^{-7}) to 3 sterile petriplates.
2. Pour approximately 15ml of molten NA medium maintained at 45°C in hot water bath to each petridish (approx 4mm height)
3. Homogenize the sample and medium by gentle rotation of petriplates, allow to settle down to solidify.
4. Incubate at 37°C for 24 hours.

Observation

S.N.	Sample	Dilution	No. of Organisms	Total no. of organisms (Dilution X no. of organism)	Average no. of organisms

Enumeration and Calculation

$$\text{No. of cfu per ml.} = \frac{\text{No. of colonies}}{\text{Dilution x 1}}$$

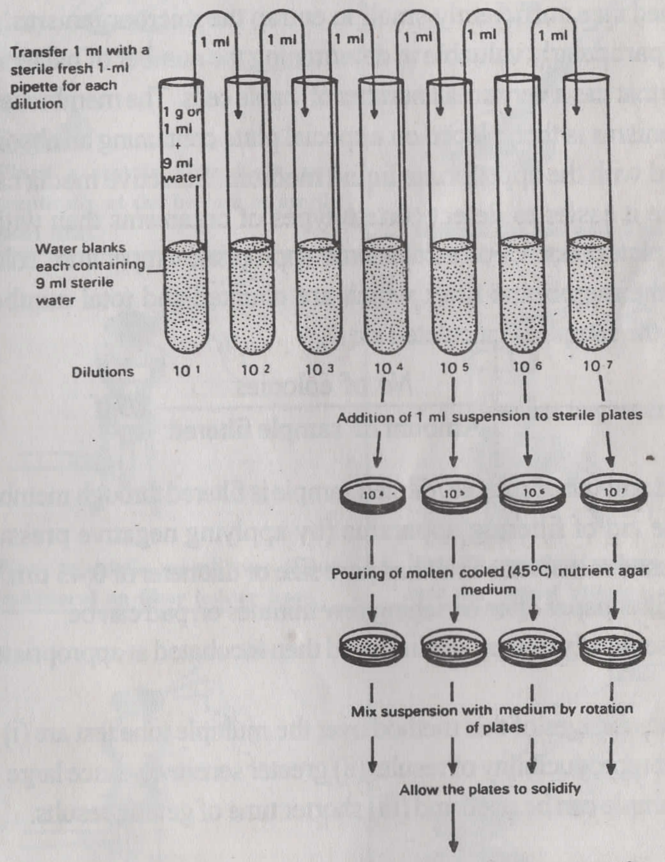
Loop Dilution Method

This method consists of diluting out one loopfull of organisms with tubes of liquified media serially in such a manner that one of the plates poured will have an optimum number of organism for dilution.

Precautions

1. The media should not be too hot
2. The sample and diluent should be shaken well.
3. The medium and sample should also be well homogenized.

[If you are using Quebec colony counters, place the plate on the counter with lid removed. Start counting at the top of the plate, using grid lines to prevent counting the same colony twice count every colony regardless of how small or insignificant. If available, use hand counter].


Transfer 1 ml with a sterile fresh 1-ml pipette for each dilution
1 ml
1 ml
1 ml
1 ml
1 ml
1 ml
1 g or 1 ml + 9 ml water
Water blanks each containing 9 ml sterile water
Dilutions
10^1
10^2
10^3
10^4
10^5
10^6
10^7
Addition of 1 ml suspension to sterile plates
10^4
10^5
10^6
10^7
Pouring of molten cooled (45°C) nutrient agar medium
Mix suspension with medium by rotation of plates
Allow the plates to solidify

Incubate at 37°c
for 24-48 hrs.

Figure 6.2 : The Pour Plate Technique (Standard Plate Count).

[If you are using electronic colony counter, place Petri plate on the illuminated stage. Press the count bar. The precise number of colonies is instantly displayed on a digital readout].

20. Counting of Organisms by Membrane Filter Technique

The use of molecular or membrane filters is a very useful variation on the plate count technique. These filters have a known uniform porosity of predetermined size sufficiently small to entrap the microorganisms. This technique is particularly valuable in determining the number of bacteria in a large sample that has a very small number of viable cells. The membrane with trapped organisms is then placed on a special plate containing an absorbent pad saturated with the appropriate liquid medium. Selective media can be used to make it easier to detect certain types of organisms than with the conventional plate count. Upon incubation at appropriate temperature, colonies develop on the membrane filter which are counted and total number of organisms in the sample is calculated as:

$$\text{No. of organisms per ml.} = \frac{No.\ \text{of colonies}}{\text{Amount of sample filtered}} xD.f$$

Fixed amount of sample /diluted sample is filtered through membrane filter with the aid of filtering apparatus (by applying negative pressure.) Extensively used membrane filter has pore size or diameter of 0.45 µm.

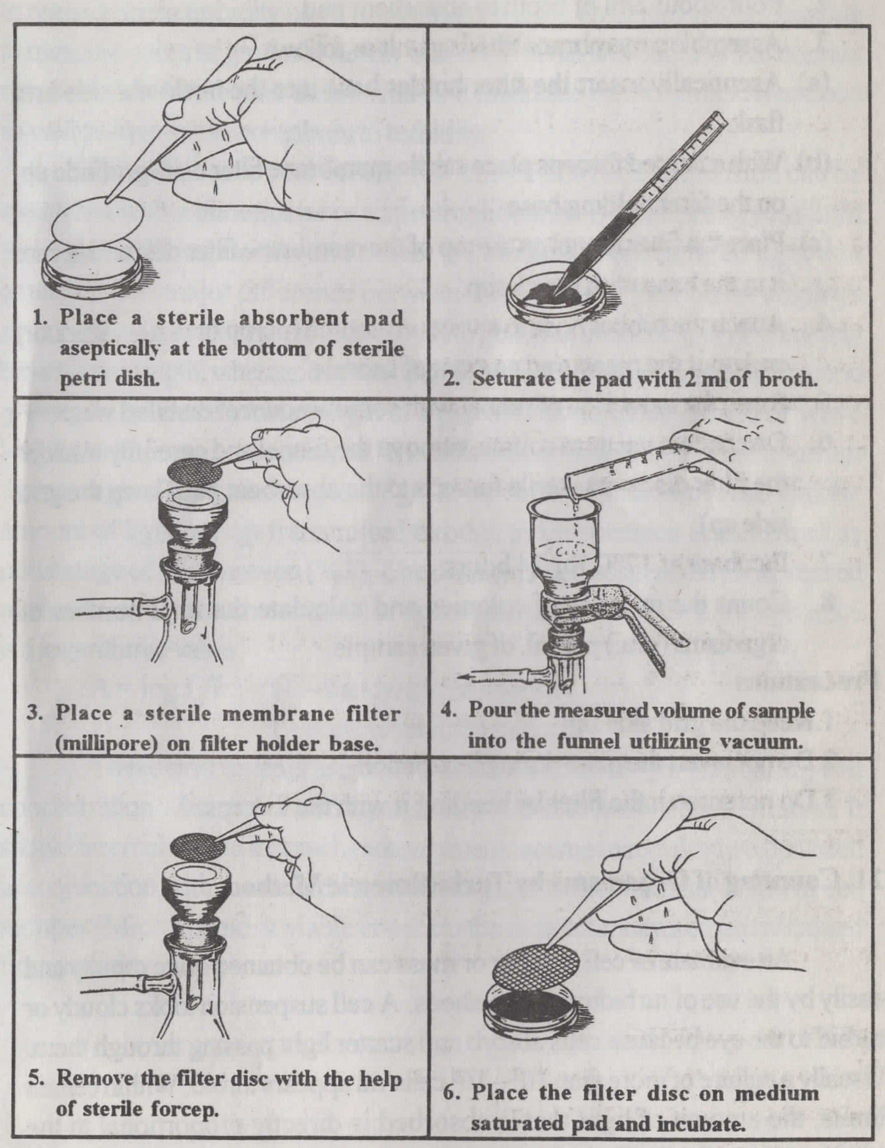
The filter paper after enriching few minutes on pad can be transferred aseptically to agar medium and then incubated at appropriate temperature.

The advantages of this method over the multiple tube test are (i) high degree of reproducibility of results (ii) greater sensitivity since large volumes of sample can be used and (iii) shorter time of getting results.

Materials Required

Vacuum pump or water faucet aspirators,
Membrane filter assemblies (sterile)
Sterile petriplates
Sterile forceps

Agar medium /broth medium eg. M-endo broth, M – endo agar
Sample



1. Place a sterile absorbent pad aseptically at the bottom of sterile petri dish.

2. Seturate the pad with 2 ml of broth.

3. Place a sterile membrane filter (millipore) on filter holder base.

4. Pour the measured volume of sample into the funnel utilizing vacuum.

5. Remove the filter disc with the help of sterile forcep.

6. Place the filter disc on medium saturated pad and incubate.

Figure 6.3 : Membrane Filter Technique.

Procedure

1. With a flamed forceps, transfer a sterile absorbent pad to a sterile petriplate.
2. Pour about 2ml of broth to absorbent pad.
3. Assemble a membrane filtering unit as follows
   (a) Aseptically insert the filter holder base into the neck of a side arm flask.
   (b) With a flamed forceps place sterile membrane filter disc, grid side up, on the filter holding base.
   (c) Place the filter funnel on the top of the membrane filter disc and secure it to the base with the clamp.
4. Attach the rubber hose to a vacuum source (pump or water aspirator) and pour the measured amount of sample.
5. Rinse the inner side of funnel with some amount of distilled water.
6. Disconnect vacuum source, remove the funnel and carefully transfer the filter disc with sterile forceps to the absorbent pad (keep the grid side up).
7. Incubate at 37°C for 24 hours.
8. Count the number of colonies and calculate the total number of organisms (cfu.) per ml. of given sample.

Precautions

1.Keep the grid side up

2.Do not invert the plate during incubation.

3.Do not scratch the filter by handing it with the forceps.

21. Counting of Organisms by Turbidimetric Method

An estimate of cell number or mass can be obtained quite rapidly and easily by the use of turbidimetric methods. A cell suspension looks cloudy or turbid to the eye because cells absorb and scatter light passing through them. Usually a culture of more than $10^7 - 10^8$ cells/ml appears turbid. Within certain limits, the amount of light that is absorbed is directly proportional to the concentration of cells. Turbidimetry is simple and rapid method of following growth especially to make counts on large number of cultures/samples.

[Quantitative plate count becomes a rather cubersome tool in that case since it requires a considerable number of glass wares and media and is time consuming too.] However, the culture to be measured must be dense enough to register some turbidity on the instrument. Moreover, it may not be possible to measure cultures grown in deeply coloured media and cultures that contain suspended material other than bacteria. It must also be recognized that dead as well as living cells contribute to turbidity.

Turbidity or optical density (i.e. cloudiness) of a suspension can be measured with a photometer or a spectrophotometer, devices that pass light through a cell suspension and detect the amount of unscattered light that emerges. The major difference between them is that a photometer employs simple filter. (Usually green, blue or red) to generate incident light of relatively broad wave length, whereas a spectrophotometer employs a prism or diffraction grating to generate incident light in a narrow but adjustable band of wave lengths to impinge on the sample. The cells suspended in the culture interrupt the passage of light allowing less light to reach the photoelectric cell and the amount of light energy transmitted through the suspension is measured as percentage of transmission (%T). The density of cell suspension is expressed as absorbance (photometer units) or optical density units (O.D.). [Absorbance is a logarithmic value

$A = \log I_0/I$ $\quad I_0$ – Incident light intensity

I – transmitted light intensity]

These instruments are convenient and accurate for estimating cell concentration when calibrated against suspension of known concentration. It should be emphasized that such measurements are meaningful only when used in conjunction with a standard curve relating some direct measurement of cell number (Microscopic or viable count) to the indirect measurement obtained from turbidity.

More sensitive instruments for measuring scattering are called nephelometers. They have the light sensing device arranged at right angles to incident beam of light and hence directly measure the scattered light. Growth of an organism can be followed by reading the turbidity of the broths inoculated with equal amount of test organism and incubated for different time periods in a series. Similarly, we can prepare standard curve correlating other direct measurements (number of bacteria, bacterial nitrogen) with turbidity. Thus, it is possible to use the convinient indirect measurement (turbidity) and convert the value to direct expression of growth.

Maerials Required:

Spectrophotometer
Cuvettes
Broth culture of bacteria
Sterile broth media, sterile pipette
Blank (Nutrient broth)
If organisms are suspended in distilled water, use distilled water as blank

Procedure

1. Turn on the spectrophotometer by rotating the zero control knob clockwise
2. Allow the instrument to warm up for 15 minutes
3. Set wavelength (e.g. at 686 nm)
4. Adjust the zero control to set % T at 0 percent (O.D to 2) by bringing knob to the left.
5. Take out the cuvette, pour blank into it, clean and dry up the cuvette, place it back to sample holder and close the lid.
6. To standardize the instrument, set % T at 100 by turning the knob to right.
7. Remove blank from sample holder.
8. Put the culture into the sample holder, close it and read % T from the scale.
9. Repeat the process for other samples too.
10. Calculate the absorbance as A = - log %T / 100 or O.D.= 2-log % T.

Precautions

1. Rinse cuvette several times with distilled water while cleaning.
2. Keep the base of cuvette spotlessly clean by keeping it free of liquids, smudges and finger prints, wipe it clean with lint free tissue.
3. Do not wipe cuvettes with towels or handkerchiefs.
4. Insert cuvette into sample holder with its index line registered with index line of the holder.
5. After the cuvette is seated, line up the index lines exactly.
6. Always close the cuvette with lid while reading the scale.
7. Select wavelength based on the colour of sample.

Section - VII

Biochemical Tests for Identification of Microorganisms

The biochemical (physiological) reactions of the microorganisms are very much important in species identification. Since all physiological reactions in organisms are catalyzed by enzymes, and since each enzyme is produced by individual gene the detection of enzyme produced, is the essential tool for species identification.

Differentiation of the principal groups of enterobacteriaceae can be accomplished on the basis of their biochemical properties. For this the IMViC series of tests which stands for **Indole, Methyl red, Voges-Proskauer** and **Citrate Utilization** can be used.

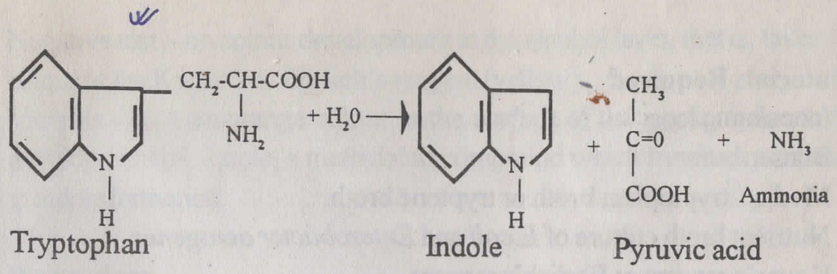
22. Tryptophan Hydrolysis (Indole Test)

The basic principle of indole test is to determine the ability of an organism to split the aminoacid "tryptophan" into Indole. This test is one of the important biochemical tests to differentiate between genera and even between species of the bacteria particularly of the enterobacteriaceae group.

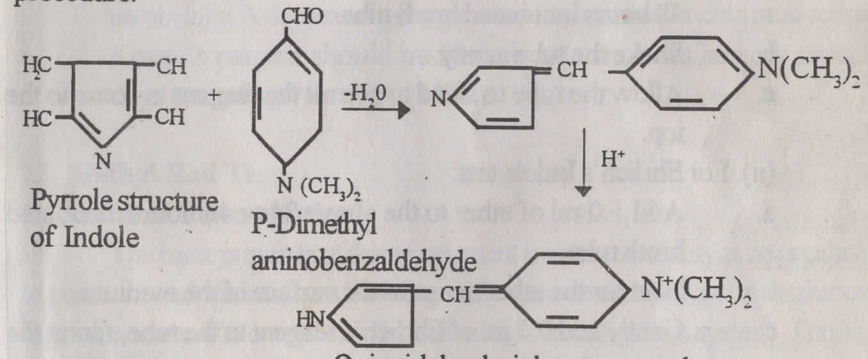
Tryptophan is an amino acid that can be oxidised by certain bacteria to form three major indolic metabolites; indole, skatole (methyl indole) and indoleacetic acid (IAA-indoleacetate). Various intracellular enzymes involved are collectively called "tryptophanase" that mediate the production of indole.

The major intermediate in tryptophan degradation is indolepyruvic acid from which indole can be formed through deamination, and skatole through decarboxylation of indoleacetic acid (IAA). The enzyme "Tryptophanase" catalyzes the deamination reaction, attacking the tryptophan molecule only in its side chain and leaving the aromatic ring intact in the form of indole.

The degradation of tryptophan releases indole, pyruvic acid, ammonia and energy. The pyruvic acid can be further metabolized either by way of the glycolytic cycle or can enter the Kreb's cycle to release CO_2, H_2O, and a large yield of energy. The NH_3 can be used to synthesize new aminoacids by utilizing the energy present for the anabolic reaction.



The indole, split from the tryptophan molecule, can be detected by a reagent which involves a chemical combination producing a distinct colour. The presence or absence of indole formation is used for bacterial identification. Indole, if present, combines with the aldehyde present in either Kovacs (or Ehrlich's) reagent to give a red colour in the alcohol layer. The colour reaction is based on the presence of the pyrrole structure present in indole. The CH_2 group of pyrrole permits condensation with the aldehyde in a two step procedure.



Quinoidal red-violet compound
(Red-deing compd)

The alcoholic layer extracts and concentrates the red colour complex. The characteristics of some of the bacteria towards indole test are:

E.coli (usually +), *Klebsiella – Entetobacter* (usually -)
Bacillus alvei (+), other *Bacillus sps.* (-)
Proteus mirabilis (-), other *Proteus sps.* (+)

Materials Required

1. Inoculating loop
2. Bunsen burner
3. Media : tryptophan broth or tryptone broth.
4. Nutrient broth culture of *E.coli* and *Enterobacter aerogenes*.
5. Kovac's reagent or Ehrlich's reagent

Procedure

1. Take a sterilized (autoclaved at 121°C at 15 lb/inch² pressure for 15 min.) test tube containing approximately 4.0 ml. of tryptophan broth.
2. Inoculate the tube aseptically by taking growth from an 18 to 24 hours pure culture with the help of sterilized inoculating loop.
3. Incubate the tube at 35°C for 24 to 48 hours.
4. (i). For Kovacs Indole test:
    a. Add 5 drops of Kovac's reagent directly to the above 24 or 48 hours incubated broth tube.
    b. Shake the tube gently.
    c. Allow the tube to stand to permit the reagent to come to the top.

    (ii). For Ehrlich's Indole test:
    a. Add 1.0 ml of ether to the above 24 or 48 hours incubated broth tube.
    b. Wait for the ether to rise to the surface of the medium.
    c. Gently add 0.5 ml of Ehrlich's reagent to the tube, tilting the tube so that the reagent runs down the side of the tube.

Observations

S.N.	Sample	Reagent used	Colour observed	Results	Remakrs
1.					
2.					
3.					

Results : For Either Reagent

Positive test :- a red ring at the surface of the medium in the alcoholic layer.

Negative test :- no colour development at the alcohol layer, that is, takes the colour of the Kovac's or Ehrlich's reagent (yellow).

Variable (±) :- an orange colour at the surface of the medium due to the development of skatole, a methylated compound which may be a precursor to indole formation.

Precautions

1. Peptone medium containing glucose must not be employed for Indole detection. The high acidity produced by glucose fermentation may prevent the growth of the organism or inhibit the enzyme. The addition of tryptophan stimulates indole production while glucose inhibits its production.
2. The optimum pH for tryptophanase activity is 7.4 to 7.8; a decrease in pH (towards acidity) results in a decrease in Indole production and a possible false negative or weakly positive reaction.
3. Cultures to be tested for Indole production must be incubated aerobically. A decrease in oxygen tension decreases indole production.
4. Kovac's reagent should be fresh; however, it can be kept several days in a refrigerator (4 to 10°C).

23. Methyl Red Test
(Mixed Acid Fermentation)

The basic principle of this experiment is to test the ability of an organism to produce and maintain sufficient stable acid end products from glucose fermentation and to overcome the buffering capacity of the system. This is a qualitative test for acid production (pH determination) since some organisms produce more acids than others.

The methyl red test is based on the use of a pH indicator, methyl red, to determine the hydrogen ion concentration (pH) present when an organism ferments glucose.

This test is basically important to differentiate between the genera of Enterobacteriaceae group. All members of the Enterobacteriaceae are glucose fermenters. In MR/VP broth medium, after 18 to 24 hours of incubation, different acidic metabolic byproducts are produced by fermentation, therefore, initially all enterics will give a positive methyl red reaction if tested. However, after further incubation, (2 to 5 days), those organisms that are methyl red positive continue to produce more acids, resulting in a low terminal pH, overcoming the phosphate buffering system and maintaining an acid environment in the medium (pH 4.2 or less). The methyl red negative organisms continue to further metabolize the initial fermentation products by decarboxylation, producing neutral acetyl methyl carbinol (acetoin), which results in a high terminal pH that decreases the medium's acidity, raising the pH toward neutrality (pH 6.0 or greater).

E.coli and other MR positive organisms produce a high yield of acids: lactic, succinic, acetic and formic; formic acid is again brokendown into hydrogen and carbondioxide. *E.coli* produces equal amounts (1:1 ratio) of H_2 and CO_2 from glucose metabolism. The overall reaction of glucose metabolism by *E.coli* is given as :

$$2\ \text{Glucose} + H_2O \rightarrow 2\ \text{lactic acid} + \text{acetic acid} + \text{ethanol} + 2CO_2 + 2H_2$$

$$\text{Formic acid} \xrightarrow[\text{hydrogenylase}]{\text{Formic}} H_2 + CO_2$$

Methyl red positive organisms produce stable acids, maintaining a high concentration of hydrogen ions until a certain concentration is reached and then all activities cease.

The reagent "Methyl Red" is an indicator which is already acid and will denote changes in degree of acidity by colour reactions over a pH range of 4.4 to 6.0. At a pH of 4.4 or less in the medium, the reagent remains red, while with a descrease in acidity and at a pH of 6.0, methyl red indicator changes to a yellow colour.

The characteristics of some of the bacteria towards MR test are: *E.coli* (+), *Enterobacter aerogenes* (-), *Klebsiella* (usually -). *Yersinia sps.* (+)

Materials Required

(1) Inoculating loop
(2) Bunsen burner

(3) Medium :- clark and Lubs medium (MR/VP Broth).
(4) Nutrient broth culture of *E.coli* and *Klebsiella sps.*
(5) Reagent :- Methyl Red pH Indicator.

Procedure

1. Take a sterilized (autoclaved at 121°C, 15 lb/inch^2 for 15 min.) tube containing approximately 5.0 ml. of MR/VP broth.
2. Inoculate the tube aseptically by taking growth (light inoculum) from an 18 to 24 hours pure culture with the help of sterilized inoculating loop.
3. Incubate the tube at 35°C for 48 hours. It is recommended to incubate at 30°C for 3 to 5 days.
4. Add 5 drops of the Methyl Red indicator directly to the incubated tube of each set *E.coli* and *Klebsiella* sps.)
5. Observe the change in colour of Methyl red.

Observations

S.N.	Sample	Reagent used	Colour observed	Results	Remakrs
1.					
2.					
3.					

Results

Positive test :- Distinct Red colour (due to pH range 4.4) at the surface of the medium.

Negative test :- yellow colour (due to pH 6.0) at the surface of the medium.

Delayed reaction:- organge colour. Continue incubation to 4 days and repeat the test.

Precautions

1. Be sure that the tubes are labelled correctly prior to inoculation, as any mix-up of media makes all test results invalid.
2. The methyl red reaction cannot be accelerated by increasing the glucose concentration in the medium.
3. No attempt should be made to interprete a methyl red result after

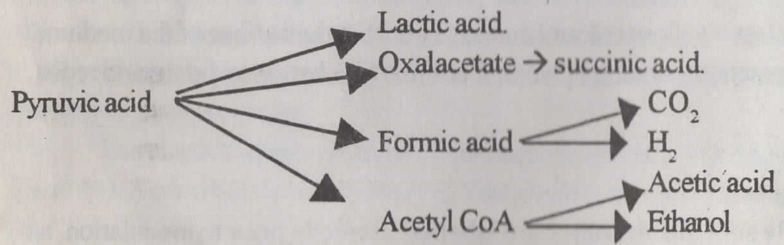
fewer than 48 hours of incubation. If the MR test is performed too early, the results are often equivocal or falsely positive since MR negative organism may not have had sufficient time to metabolize completely the initial acidic products that accumulated from glucose fermentation.

4. Avoid testing an extremely turbid broth-inoculum mixture; bacterial growth is inhibited if the inoculum exceeds the maximum cell concentration of about 10^9 viable cells/ml.

24. Voges-Proskauer Test

The basic principle of this test is to determine the ability of some organisms to produce a neutral end product, acetylmethyl carbinol (acetoin $CH_3.CO.CHOH.CH_3$) from glucose fermentation. Glucose is metabolized to pyruvic acid which is the key intermediate in glycolysis. From pyruvic acid, an organism may follow many pathways. The production of acetoin is one pathway for glucose degradation occurring in bacteria.

The voges-proskauer test for acetoin is used primarily to differentiate *E.coli* from the *Klabsiella-Enterobacter* groups although other members of the Enterobacteriaceae are capable of producing a positive VP. The bacteria of Enterobacteriaceae are characteristically classified as mixed acid or formic acid fermenters indicating that their end products of glucose fermentation are acidic: formic acid, acetic acid, succinic acid, ethanol, hydrogen and carbon dioxide.



These mixed acid fermenter can be further divided into two groups (i) those that produce acids but no 2,3 butanediol (or 2,3 butylene glycol), such as *E.coli* (VP-) or, (ii) those that produce 2,3 butanediol as their major end products such as *Klebsiella-Enterobacter* groups (VP+)

The major end product of pyruvate utilization by the *Klebsiella-Enterobacter* groups, *Serratia, Bacillus*, and many other micro-organisms, is 2,3-butanediol. However, the VP test is based on the detection of acetoin (acetylmethyl carbinol), a precursor to 2,3-butanediol production. One molecule of acetoin is formed by the decarboxylation of two molecules of pyruvic acid. Acetoin is an intermediate stage in the conversion to 2,3-butanediol. Both acetoin and 2,3 butanediol are neutral products from glucose fermentation.

2 Pyruvate → acetoin + $2CO_2$

The acetoin and 2,3 butanediol formation is a reversible reduction or oxidation system, which can be represented as-

$$\text{2,3 Butanediol} \xrightarrow[\text{2,3 butanediol dehydrogenase}]{\text{Oxidation}} \text{Acetoin}$$

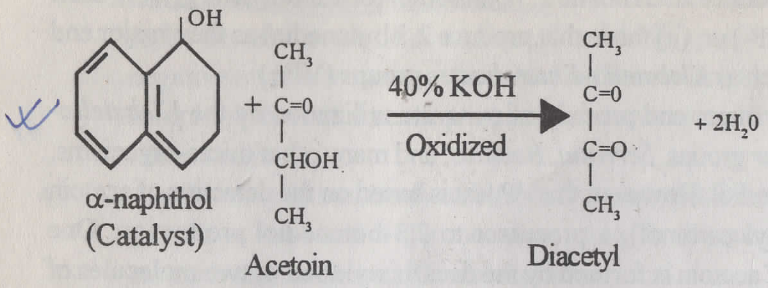
$$\text{Acetoin} \xrightarrow[\text{diacetyl acetoin reductase}]{\text{reduction}} \text{2,3 butanediol}$$

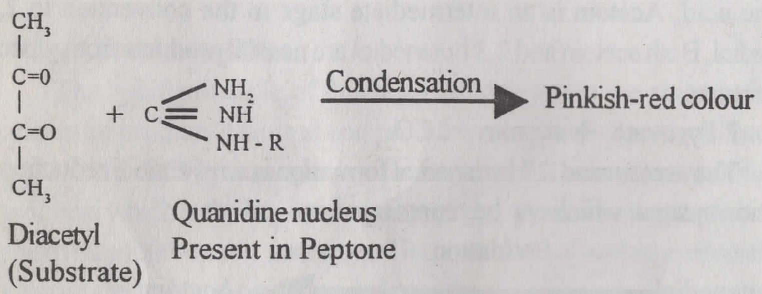
In the presence of atmospheric oxygen and alkali, the neutral end products "acetoin and 2,3 butanediol" are oxidized to diacetyl, which is the reactant for the colour produced in the VP test. Therefore, diacetyl is an oxidation product of acetoin.

The overall reaction of glucose metabolism by the *Klebsiella – Enterobacter* groups follows-

Glucose → 2,3 butanediol + $2CO_2$ + H_2.

For detection of acetoin (acetylmethyl carbinol) in VP test, Barritt's reagent is used which acts in a two step procedure-





The VP test is based on the reaction between diacetyl and the quanidine nucleus (NH:C(NH_2). NH.R) present in peptone under alkaline conditions.

The first reagent added to an incubated aliquot is the catalyst α-naphthol. An alcoholic solution of α-naphthol is essential in that it acts as a colour intensifier. The α-naphthol combines with the product of the reaction of diacetyl and the quanidine type compound arginine present in peptone early in the reaction. The second reagent is 40% KOH which when added to the VP medium, aids in the absorption of CO_2. Potassium hydroxide acts as an oxidizing agent to hasten the oxidation of acetoin to diacetyl. Diacetyl is the essential reactant that gives a colour reaction with KOH and the peptone.

Therefore, when diacetyl, peptone, α-naphthol and KOH are mixed in correct quantities, a red colour develops almost instantly at the surface of the medium. The rate of colour development depends on the amount of acetoin present in the test culture. A positive reaction usually occurs within 2 to 5 minutes exhibiting a faint pink colour, however, a marked colouration (a deep red) occurs upon standing for 30 minutes.

The characteristics of some of the bacteria towards VP test are- *Klebsiella pneumoniae* (+), *Enterobacter* (usually +) *E.coli* (-)

5% α-napthol in ethyl alcohol

Materials Required

(1) Inoculating loop
(2) Bunsen burner
(3) Medium :- Clark and Lubs Medium (MR/VP Broth) pH 6.9.
(4) Nutrient broth culture of *E.coli* and *Klebsiella sps.*
(5) Reagents :- Barrit's reagent.
    (a) α-Naphthol 5% in ethyl alcohol
    (b) 40% KOH solution

Procedure

1. Take a serilized (autoclaved at 121°C, 15 lb pressure for 15 min.) tube containing approximately 5.0 ml of MR/VP broth.
2. Inoculate the tube with light inoculum of test organisms from the growth of an 18 to 24 hours pure culture with the help of inoculating loop.
3. Incubate the tube at 35°C for 24 to 48 hours. If *Enterobacter hafniae* is suspected, repeat the VP culture and incubate at 25°C, since this organism is VP variable at 37°C.
4. Aseptically take 2.5 ml. of incubated test culture in a tube for VP test. (Half portion of test culture can be used for MR test).
5. Add 0.6 ml. of a 5% α-naphthol solution first to the above tube.
6. Add 0.2 ml. of 40% KOH solution to the above tube.
7. Shake the tube gently to expose the medium to atmospheric oxygen in order to oxidize the acetoin so as to obtain a colour reaction.
8. Allow tube to stand at least 10 to 15 minutes for observation.

Observations

S.N.	Sample	Reagent used	Colour observed	Results	Remakrs
1.					
2.					
3.					

Results

VP positive test :- pinkish – red colour at the surface of the medium (Presence of acetoin).

VP negative test :- yellow colour at the surface of the medium (same as the colour of reagent). A copper like colour may form but it is still a negative reaction (due to action of reagents when mixed.)

Precautions

1. Be sure that the tubes are labeled correctly prior to inoculation, as any mix-up of media makes all test results invalid.
2. The order of adding Barrit's VP reagents is extremely important. First add α-Naphthol, followed by KOH. A reversal of the order of reagent addition may give a weak positive or false negative result.
3. An exact amount, 0.2 ml. of 40% KOH should not be exceeded, since, excess KOH may mask a weak VP positive reaction by exhibiting a copper like colour due to its reaction with α-Naphthol alone.
4. Occasionally, a known acetoin positive organism fails to give a positive VP reaction. To overcome this possibility, gently heat the culture containing the VP reagents. A VP positive organism will show a positive test (red colour), while those organisms not producing acetoin will remain negative (a yellow colour).

25. Citrate Utilization Test

The basic principle of citrate utilization test is to determine the ability of an organism to utilize citrate as the sole source of carbon for metabolism with resulting alkalinity.

Normally, citrate metabolism involves a condensation of acetyl, with coenzyme A and oxaloacetate to enter the kreb's cycle. Citrate metabolism by most bacteria is rapid via the tricarboxylic acid cycle or the citrate fermentation pathway. In bacteria the cleavage of citrate involves an enzyme system without the intervention of the coenzyme A. This enzyme is called citratase (citrate oxaloacetate-lyase) or citrate demolase. The enzyme requires a divalent cation for its activity which is supplied by magnesium or manganese. Thus, the utilization of citrate depends on the presence of enzyme produced by organisms that breaks down the citrate to oxaloacetic acid and acetic acid as an intermediate products. These products are later converted to pyruvic acid and carbon dioxide.

$$\begin{array}{l} \text{Citrate} \rightarrow \text{oxaloacetate} + \text{acetate} \\ \qquad\qquad\qquad \downarrow \\ \qquad\qquad\quad \text{Pyruvate} + CO_2 \end{array}$$

The medium used in this experiment is Simmon's citrate agar which contains sodium citrate as the only source of carbon and energy. The indicator used in it is bromothymol blue. When the citrate is metabolized by the organisms, the CO_2 generated combines with sodium and water to form sodium carbonate, which is an alkaline product. The carbonate so produced changes the colour of the indicator from green to blue which is a positive test.

$$CO_2 + 2Na^+ + H_2O \rightarrow \underset{\text{(Alkaline)}}{Na_2CO_3}$$

The medium used for citrate fermentation also contains inorganic ammonium salts. An organism that is capable of utilizing citrate as its sole source of carbon, also utilizes the ammonium salts as its sole nitrogen source. Ammonium salts are broken down to ammonia (NH_3) with resulting alkalinity.

The characteristics of some of the bacteria towards citrate utilization test are:

E.coli (-), *Klebsiella sps.* (usually +), *Enterobacter sps.* (+), *Edwardsiella sps.* (-), *Salmonella sps.* (usually +).

Materials Required

(1) Inoculating needle
(2) Bunsen burner
(3) Medium :- Simmon's citrate agar slants (Alternatively christensens citrate medium can be used which contains phenol red as an indicator)
(4) Nutrient broth cultures of *E.coli* and *Klebsiella sps.*

Procedure:

1. Take a sterilized (autoclaved at 121°C at 15 lb for 15 minutes) tube containing 4 to 5 ml. of medium (long slant, short butt).
2. Inoculate light inoculum from the growth of an 18 to 24 hours pure culture of test organism by means of a stab-and-streak inoculation.

colour of reagent). A copper like colour may form but it is still a negative reaction (due to action of reagents when mixed.)

Precautions

1. Be sure that the tubes are labeled correctly prior to inoculation, as any mix-up of media makes all test results invalid.
2. The order of adding Barrit's VP reagents is extremely important. First add α-Naphthol, followed by KOH. A reversal of the order of reagent addition may give a weak positive or false negative result.
3. An exact amount, 0.2 ml. of 40% KOH should not be exceeded, since, excess KOH may mask a weak VP positive reaction by exhibiting a copper like colour due to its reaction with α-Naphthol alone.
4. Occasionally, a known acetoin positive organism fails to give a positive VP reaction. To overcome this possibility, gently heat the culture containing the VP reagents. A VP positive organism will show a positive test (red colour), while those organisms not producing acetoin will remain negative (a yellow colour).

25. Citrate Utilization Test

The basic principle of citrate utilization test is to determine the ability of an organism to utilize citrate as the sole source of carbon for metabolism with resulting alkalinity.

Normally, citrate metabolism involves a condensation of acetyl, with coenzyme A and oxaloacetate to enter the kreb's cycle. Citrate metabolism by most bacteria is rapid via the tricarboxylic acid cycle or the citrate fermentation pathway. In bacteria the cleavage of citrate involves an enzyme system without the intervention of the coenzyme A. This enzyme is called citratase (citrate oxaloacetate-lyase) or citrate demolase. The enzyme requires a divalent cation for its activity which is supplied by magnesium or manganese. Thus, the utilization of citrate depends on the presence of enzyme produced by organisms that breaks down the citrate to oxaloacetic acid and acetic acid as an intermediate products. These products are later converted to pyruvic acid and carbon dioxide.

$$\begin{tikzcd} \text{Citrate} \arrow[r] & \text{oxaloacetate} + \text{acetate} \arrow[d] \\ & \text{Pyruvate} + CO_2 \end{tikzcd}$$

The medium used in this experiment is Simmon's citrate agar which contains sodium citrate as the only source of carbon and energy. The indicator used in it is bromothymol blue. When the citrate is metabolized by the organisms, the CO_2 generated combines with sodium and water to form sodium carbonate, which is an alkaline product. The carbonate so produced changes the colour of the indicator from green to blue which is a positive test.

$$CO_2 + 2Na^+ + H_2O \rightarrow \underset{\text{(Alkaline)}}{Na_2CO_3}$$

The medium used for citrate fermentation also contains inorganic ammonium salts. An organism that is capable of utilizing citrate as its sole source of carbon, also utilizes the ammonium salts as its sole nitrogen source. Ammonium salts are broken down to ammonia (NH_3) with resulting alkalinity.

The characteristics of some of the bacteria towards citrate utilization test are:

E.coli (-), *Klebsiella sps.* (usually +), *Enterobacter sps.* (+), *Edwardsiella sps.* (-), *Salmonella sps.* (usually +).

Materials Required

(1) Inoculating needle
(2) Bunsen burner
(3) Medium :- Simmon's citrate agar slants (Alternatively christensens citrate medium can be used which contains phenol red as an indicator)
(4) Nutrient broth cultures of *E.coli* and *Klebsiella sps.*

Procedure:

1. Take a sterilized (autoclaved at 121°C at 15 lb for 15 minutes) tube containing 4 to 5 ml. of medium (long slant, short butt).
2. Inoculate light inoculum from the growth of an 18 to 24 hours pure culture of test organism by means of a stab-and-streak inoculation.

3. Incubate the inoculated tube at 35°C for 24 to 48 hours.
4. Incubate an uninoculated slant tube for comparative control in the same way.

Observations: Observe the slant cultures for the growth and colouration of the medium.

S.N.	Sample	Media used	Initial Colour	Colour Changed	Result	Rermarks
1.						
2..						
3.						

Results

Citrate positive test :- growth with an intense blue colour on the slant.
Citrate negative test :- no growth with no change in colour (green).

Precautions

1. When inoculating a series of biochemicals from the same culture, either flame the inoculating needle before streaking citrate medium or inoculate it first. Any carry-over of glucose or other nutrients onto the citrate medium can result in a false positive test.
2. Always use a light inoculum. A too heavy inoculum may give a false positive result.

26. Catalase Test

The basic principle of catalase test is to determine the presence of "Catalase" an enzyme in micro-organism that decomposes hydrogen peroxide to release oxygen.

The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria-with the main exception of *Streptococcus sps.* Usually organisms which lack the cytochrome system also lack the catalase enzyme and therefore are unable to break down hydrogen peroxide.

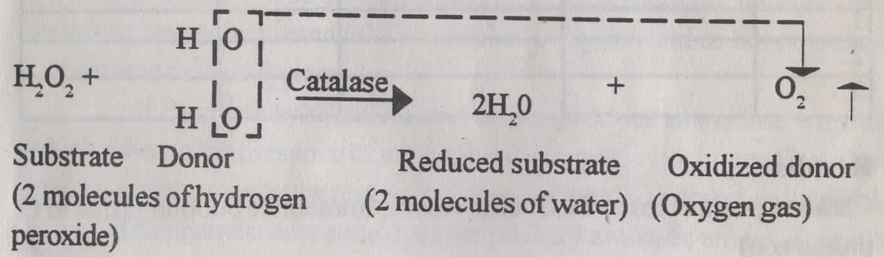
Catalase is a hemeprotein, the prosthetic group of it is made up of four atoms of trivalent iron (ferric-Fe^{+++}) per molecule, which retains its oxidized state during enzyme activity.

In microorganism, Hydrogen peroxide is formed as an oxidative end product of the aerobic breakdown of sugars. Reduced flavoprotein reacts directly with gaseous oxygen by way of electron reduction to form hydrogen peroxide and not by direct action between hydrogen and molecular oxygen.

$$\underset{\text{Reduced Flavoprotein}}{FPH_2} + O_2 \longrightarrow \underset{\text{Oxidized Flavoprotein}}{FP} + H_2O_2$$

Hydrogen peroxide, if allowed to accumulate, is toxic to bacteria, resulting in their death. It has been postulated that the death of strict anaerobes in the presence of oxygen is due to the suicidal act of H_2O_2 production in the absence of catalase production.

In the decomposition of hydrogen peroxide, one molecule acts as the substrate and the other as a donor, the substrate reduced by hydrogen atoms supplied by the donor results in a reduced substrate (H_2O) and an oxidized donor (O_2)



The catalase test is primarily used to differentiate between genera: *Streptococcus* (-) from *Micrococcus* (+) and *Staphylococcus* (+), *Bacillus* (+) from *Clostridium* (-)

Materials Required

(1) Inoculating needle (Nichrome wire)
(2) Glass slide
(3) Bunsen burner
(4) Cultures of *Streptococcus lactis* and *Staphylococcus aureus*
(5) Pure agar slant culture of test organism

(6) Hydrogen peroxide (3%)

Procedure

(A) Slide Method

(1) Pick the centre of an 18 to 24 hours pure colony with the help of an inoculating needle (Nichrome wire) and place on a clean glass slide.

(2) Add a drop of 3% H_2O_2 over organism on slide with the help of a dropper or Pasteur pipette.

(3) Observe for immediate bubbling (gas liberation).

(B) Tube Method

(1) Directly add 1.0 ml. of 3% H_2O_2 to an 18 to 24 hours heavily inoculated pure nutrient agar slant culture.

(2) Observe for immediate bubbling (gas liberation)

Observations

S.N.	Sample	Reagent used	Results	Remarks
1.				
2.				
3.				

Results

Catalase positive test :- H_2O_2 effervesces, immediate bubbling (due to O_2 production)

Catalase Negative test :- No bubbling (no O_2 formed)

Precautions

1. The test organism should not be taken from blood agar culture. Red blood cells contain catalase and their presence will give a false positive result.
2. Growth for catalase testing must be from an 18 to 24 hours culture. Older colonies may lose their catalase activity, possibly resulting in a false negative reaction.
3. Hydrogen peroxide must be fairly fresh as it is very unstable and easily breaks down on exposure to light. The solution must be kept refrigerated at all times when not in use.

27. Oxidase Test

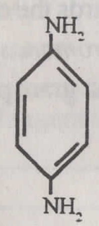
The basic principle of this test is to determine the presence in bacteria of oxidase enzyme that will catalyse the transport of electrons between electron donors in the bacteria and a redox dye-tetramethyl-p-phenylene-diamine.

The oxidase reaction is due to the presence of a cytochrome oxidase system-which activates the oxidation of reduced cytochrome by molecular oxygen, which in turn acts as an electron acceptor in the terminal stage of the electron transfer system.

During respiration, molecular oxygen oxidizes a substrate through the intervention of the electron transport system. The cytochrome system is usually present only in aerobic organisms which makes them capable of utilizing oxygen as a final hydrogen acceptor to reduce molecular oxygen to hydrogen peroxide. All oxidase positive organisms are either aerobic or facultative anaerobic, with the exception of *Vibrio fetus* which requires a microaerophilic oxygen requirement. A positive oxidase reaction is also limited to those organisms capable of growing in the presence of oxygen and at the same time producing the enzyme catalase. Obligate anaerobes lack oxidase activity since they are unable to live in the presence of atmospheric oxygen and do not possess a cytochrome oxidase system.

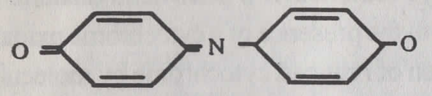
All *Pseudomonas* and *Neisseria sps.* produce an oxidase enzyme which when in the presence of atmospheric oxygen, cytochrome C, and an oxidase reagent, oxidize the reagent to form a coloured compound, indophenol.

The phenylenediamine dyes are primary aromatic amines diamino derivatives of benzene.

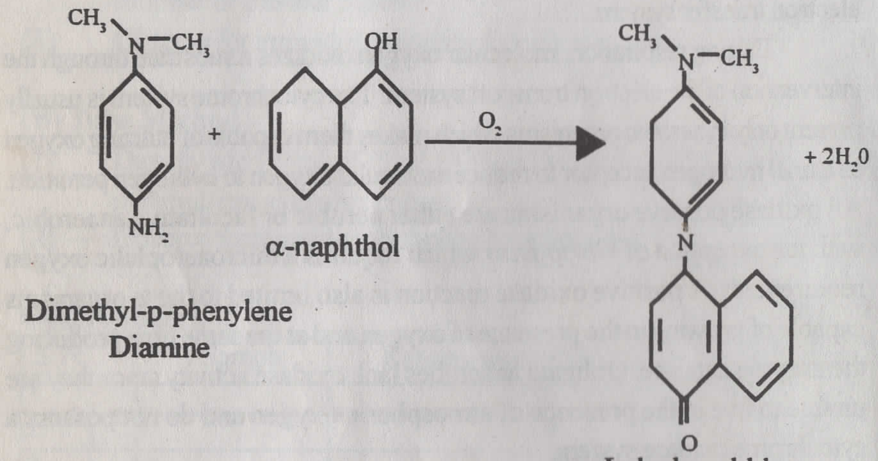


p-phenylenediamine

Cytochrome oxidase, in the presence of atmospheric oxygen, oxidizes the phenylenediamine oxidase reagent to form a coloured compound, indophenol. If the oxidase reagent is prepared with α-naphthol, indophenol blue is formed, however, α-naphthol is not necessary for the reaction to take place.



Indophenol



Dimethyl-p-phenylene Diamine

α-naphthol

Indophenol blue

The reagent phenylenediamine hydrochloride forms a dichlorimide group ($ClN=C_6H_4=NCl$); the $=NCl$ undergoes either reduction to $-NH_2$ or hydrolysis to $=0$

The characteristics of some of the bacteria towards the oxidase test are: *Pseudomonas* (usually +), *Alcaligenes* (+), *Aeromonas* (+), *Neisseria* (+), *Enterobacteriaceae* (-), *Acinetobacter* (-), Most gram positive bacteria are oxidase negative.

Materials Required

(1) Culture plate

(2) Platinum loop or glass rod

(3) Culture of *E.coli* and *Pseudomonas aeruginosa*

(4) Reagent :-

(a) Kovac's reagent:- 1% tetramethyl-p-phenylenediamine dihydrochloride. or

(b) Gordon and McLeod's reagent:- 1 to 1.5% dimethyl-p-phenylene diamine dihydrochloride. or

(c) Oxidase impregnated discs.

Procedure

(A) Wet-filter paper method

1. Place a piece of filter paper (Whatman's No.1) in a clean dry petridish and add 2 or 3 drops of freshly prepared oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride solution).
2. Using a piece of stick or glass rod (not an oxidized wire loop), remove a colony of the test organism and smear it on the filter paper.
3. Look for the development of a blue-purple colour within a few seconds (5-10 sec.), a "delayed positive" reaction will be indicated by colouration in 10-60 seconds.

(B) Dry filter paper method

In the case of filter paper soaked with oxidase reagent and freeze dried is used,

1. Take a filter paper strip in a clean petridish and moist it with distilled water.
2. Pick up the colony to be tested with a glass rod and smear it over the moist area of strip.
3. Observe the colour development as for the wet-filter paper method.

Observations:

S.N.	Sample	Reagent used	Initial Colour	Colour Observed	Result	Rermarks
1.						
2.						
3.						

Results

Oxidase positive test :- Blue-purple colour within 10 seconds.

A positive reaction within 10 to 60 seconds is considered a delayed result.

Oxidase Negative test :- No blue-purple colour.

Precautions

1. Acidity inhibits oxidase enzyme activity. Therefore, Do not attempt to perform an oxidase test on any colonies growing on medium containing glucose, otherwise false negative test may result.
2. The oxidase reagents quickly auto-oxidize and lose their sensitivity. The auto-oxidation is reduced by adding a 0.1% solution of ascorbic acid directly to the reagent during its preparation.

28. Nitrate Reduction Test

The basic principle of this test is to determine the ability of an organism to reduce nitrate to nitrite or free nitrogen gas by the enzyme "nitrate reductase".

The reduction of nitrate (NO^-_3) to nitrite (NO^-_2) and to nitrogen gas (N_2) usually takes place under anaerobic conditions, in which an organism derives its oxygen from nitrate. The oxygen serves as a hydrogen acceptor, that is, the final proton and electron acceptor. Most aerobic bacteria are facultative anaerobes and can only reduce nitrate in the absence of oxygen. This anaerobic respiration is an oxidation process whereby inorganic sybstances, mainly nitrate and sulfate, or rarely, carbonate furnish oxygen to serve as electron acceptors to provide energy. In nitrate reductions, bacterial cytochromes transport electrons to specific acceptor molecules.

Nitrate to Nitrite Reduction,-

$$\underset{\text{Nitrate}}{NO_3} + 2e^- + 2H^+ \xrightarrow{\text{Nitratase}} \underset{\text{Nitrite}}{NO_2} + H_2O$$

The end product possibilities of nitrate reduction are nitrite (NO_2), ammonia (NH_3), molecular nitrogen (N_2), nitric oxide (NO), nitrous oxide (N_2O) or hydroxylamine (R.NH.OH). The end product of reduction formed depends upon bacterial species. The more common end product is molecular nitrogen gas by way of nitrite reduction.

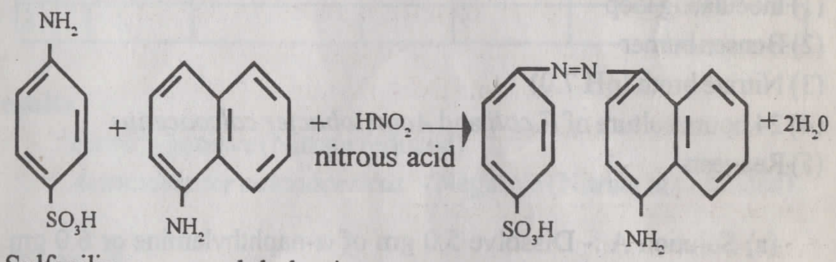
The reduction of nitrate to nitrogen gas or Nitrous oxide is called denitrification.

Nitrate to Molecular nitrogen,-

$$2NO_3^- + 10e^- + 12\,H^+ \rightarrow N_2 + 6H_2O$$

In the denitrification process, nitrous oxide (an intermediate) may accumulate if the concentration of nitrate is high, however, when the concentration of nitrate is low, nitrous oxide is further reduced to molecular nitrogen.

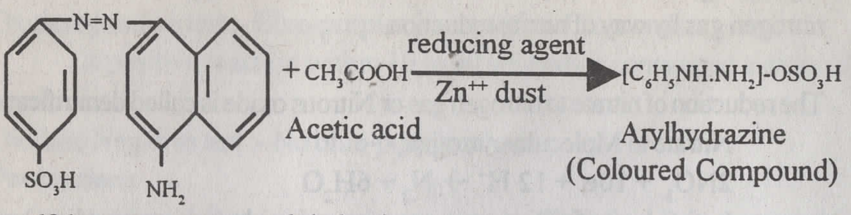
The reduction of nitrate (NO_3^-) to nitrite (NO_2^-) is denoted by a colour (red) development when nitrite reacts with the two reagents – sulfanilic acid and α-naphthylamine. The resulting colour reaction is due to the formation of a diazonim compound, P-sulfobenzene-azo-α-nepthylamine.



Sulfanilic acid (Colourless) + α-naphthylamine (Colourless) → P-sulfobenzene-azo-α-naphthylamine (red)

The –N=N-azo group linkage results in a coloured compound via a nitroso reaction.

When nitrite is not detected it is necessary to test whether the organism has reduced the nitrate beyond nitrite. This is done indirectly by checking whether the broth still contains nitrate. Zinc dust is added which will convert any nitrate to nitrite. Reduction of the diazonium salt (P-sulfobenzene-azo-α-naphthylamine) by the reducing agent zinc dust in the presence of acetic acid produces a coloured compound, arhydrazine.



P-sulfobenzene-azo-α-naphthylamine
(Diazonium Coloured Compound)

If no nitrite is detected when the zinc dust is added, it can be assumed that all the nitrate has been reduced beyond nitrite to nitrogen gas or ammonia by a nitrate reducing organism.

Materials Required

(1) Inoculating loop
(2) Bunsen burner
(3) Nitrate broth (pH 7.0)
(4) 24 hours culture of *E.coli* and *Acinetobacter calcoacetius*
(5) Reagents

(a) Solution A :- Dissolve 5.0 gm of α-naphthylamine or 6.0 gm. of dimethyl-α-napthylamine in 1 litre of acetic acid 5 mol/litre.
(b) Solution B :- Dissolve 8.0 gm of sulphanilic acid in 1 litre of acetic acid 5 mol/litre.

Immediately before use, mix equal volume of solution 'A' and solution 'B' to give the test reagent.

Procedure

1. Take a sterilized (autoclaved at 121°C, 15 lb pressure, 15 min.) tube containing Nitrate broth 1.0 ml. per tube and inoculate a heavy inoculum of growth from an 18-24 hours pure culture of test organism. or Take a sterilized Nutrient agar (5.0 ml. per tube) slant medium and inoculate as fishtail slant and stab butt.
2. Incubate the tubes at 35°C for 12 to 24 hours rarely prolonged incubation upto 4 days (96 hrs.) may be required.

3. (a) Phase -1 :- To an incubated nitrate culture, directly add 0.1 ml of the test reagent. A red colour developing within 30 sec. indicates a completed test. If negative (no colour development), proceed to phase-2.

   (b) Phase-2 (Zinc reduction method) → To the tube containing reagents A and B, directly add a pinch (approx. 20 mg) of zinc. dust (zn). Zinc dust must be nitrate-nitrite free. Observe for final interpretation. Colour develops within 30 sec.

Observations:

S.N.	Sample	Reagent used	Initial Colour	Colour Changed	Result	Rermarks
1.						
2.						
3.						

Results

E.coli :- positive (Nitrate reduced)
Acinetobacter calcoaceticus :- Negative (Nitrate not reduced).

1. Phase - 1

Positive test :- Pink to a deep red colour within 30 seconds indicating that nitrate (NO$_3^-$) is reduced to nitrite (NO$_2^-$) by organism.

Negative test :- No colour development. Proceed to phase-2.

2. Phase – 2 – Zinc reduction test :

Positive–test :- No colour development, which indicates the absence of nitrite (NO$_2^-$) in the medium. Organism reduced nitrate (NO$_3^-$) to nitrite (NO$_2^-$) and then further reduced nitrite.

Negative test :- Pink to a deep red colour which indicates the presence of nitrate (NO$_2^-$) not reduced by organism. Zinc reduced nitrate (NO$_3^-$) to nitrite (NO$_2^-$).

Precautions

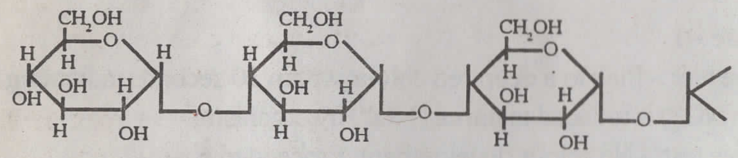
1. Dimethyl-α-naphthylamine is carcinogenic. Avoid skin contact.

2. When performing the nitrate reduction test using α-nephthylamine, the colour produced in a positive reaction may fade quickly. Therefore, the results should be interpreted immediately.
3. Eliminate fading or destruction of colour development by hydrogen sulphide (H_2S) producing organism by utilizing a sulfur free peptone medium.
4. In all cultures where nitrite is absent, when using α-naphthylamine (or dimethyl-α-naphthylamine) and sulfanilic acid reagents, add zinc dust directly to tube before making a final interpretation.

29. Starch Hydrolysis Test

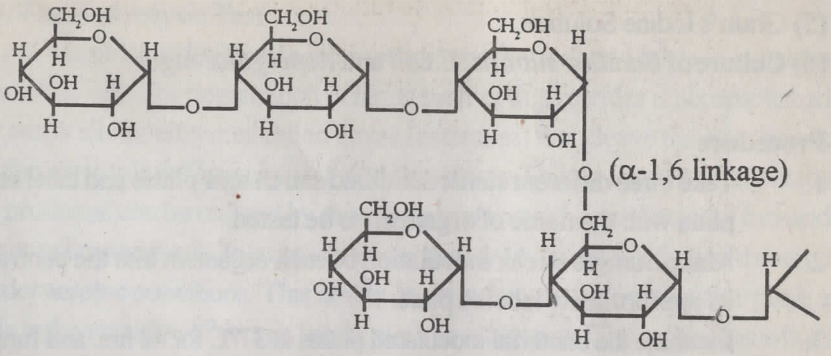
The main purpose of this test is to determine the ability of an organism to hydrolyse the starch by an exoenzyme amylase.

Starch is a complex carbohydrate (polysaccharide) which consists of a mixture of amylose and amylopectin in the proportion of 1:4. Amylose is soluble in hot water (60-80°C) whereas amylopectin is not soluble in hot water. Amylose has mol. weight from 10,000 to 50,000 (60-300 glucose units). Amylose is a continuous unbranched chain of glucose unit joined by α-(1,4) glycoside bonds. It gives intense blue colour with iodine.



[α-(1,4) glycoside bond]

Amylopectin has mol. wt. between 50,000 – 100,000 (300-600 glucose units). It has highly branched structure. It contains the same type of respective sequence of α-(1,4) glycoside bonds as does amylose but chain length vary only from about 24-30 units. In addition, there is considerable branching from this linear network. At branched point, a new chain is started by an α-(1,6) glycoside linkage between C-1 of one glucose unit and C-6 of another glucose unit.



Amylase is an exoenzyme that hydrolyses starch into maltose, a disaccharide and some monosaccharides such as glucose. These disaccharides and monosaccharides enter into the cyloplasm of the bacterial cell through the semipermeable membrane and thereby used by the endoenzymes.

Amylase production is known in some bacteria while well-known in fungi. Amylases commercially produced from various *Aspergilli* are used in the initial steps in several food fermentation processes to convert starch to fermentable sugars.

In the laboratory, the absence or presence of starch in the medium is determined by using iodine solution as an indicator. When iodine comes in contact with a medium containing starch, it turns blue. If starch is hydrolyzed and starch is no longer present, the medium appears clear (uncoloured).

The blue colour is due to the formation of an inclusion complex between iodine and the amylose fraction of starch. In forming this complex, the linear amylose molecule coils into a spiral that is held by hydrogen bonding between hydroxyl groups of adjacent loops. The iodine molecules then align themselves in the centre of the tube formed by the spiral. This arrangement allows the interaction of the electron orbitals of many iodine atoms, which causes the light absorption that gives the complex its characteristic colour.

Materials Required

(1) Inoculating loop
(2) Bunsen burner
(3) Dropper
(4) Sterile starch agar medium plate

(5) Gram's Iodine Solution

(6) Culture of *Bacillus subtilis, E.coli* and *Aspergillus niger*.

Procedure

1. Take three different sterile solidified starch agar plates and label each plate with the name of organism to be tested.
2. Make a single streak inoculation of each organism into the centre of its appropriately labeled plate.
3. Incubate the bacterial inoculated plates at 37°C for 48 hrs. and fungal inoculated plates at 28°C for 72-96 hours in an inverted position .
4. After incubation, flood the surface of the plates with iodine solution with the help of a dropper for about 30 seconds.
5. Pour off the excess iodine solution .

Observation : Examine the plates for starch hydrolysis around the line of growth of each organism, that is, the colour change of the medium.

S.N.	Organisms	Colour of the medium	Colour around line of growth	Results	Remarks
1.	*B.subtilis*				
2.	*E.coli*				
3.	*A.niger*				

Results

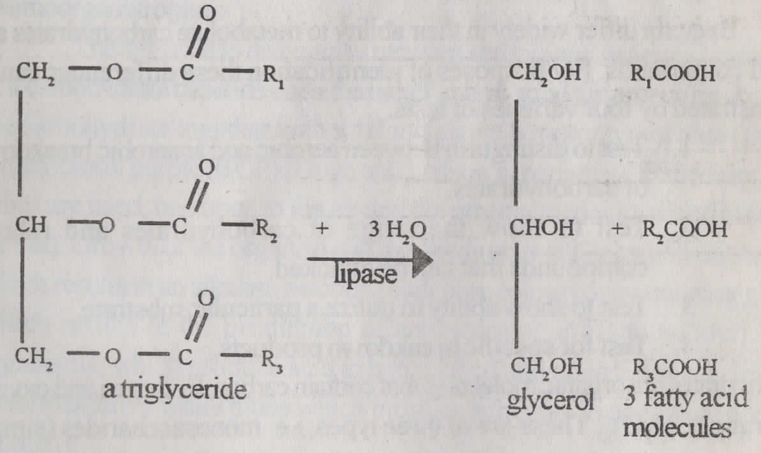
Positive test :- clear zone (transparent) surrounding the microbial colonies after addition of iodine solution indicating that the starch is hydrolysed by *B. subtilis* and *Aspergillus niger* by the production of exoenzyme amylase.

Negative test :- Dark blue colouration of the medium after addition of Iodine indicating that starch is not hydrolysed by *E.coli* i.e., absence of amylase in *E.coli*.

30. Fat Hydrolysis Test

Lipids are high molecular weight compounds, too large to enter the microbial cell. The degradation of lipids such as triglycerides is accomplished by extracellular enzymes called lipase (esterases) that cleave the ester bonds in this molecule to form glycerol and fatty acids. The glycerol and fatty acids so produced can be utilized by the organisms to synthesize bacterial fats and other cell components. In many instances they are even oxidized to yield energy under aerobic conditions. This ability of organisms to decompose fats plays a role in the rancidity of certain foods, such as margarine. The organisms which have the ability to hydrolyse the lipids are called lipolytic organisms.

In this experiment, tributyrin agar (Nutrient agar supplemented with the triglyceride tributyrin) is used. Tributyrin forms an emulsion when dispersed in the agar, producing an opaque medium which is necessary for observing lipolytic activity.



Materials Required

Inoculating loop,
Bunsen burner,
Tributyrin agar plate
Culture of *S. aureus, B. cereus*.

Procedure:

1. Take a dry Tributyrin agar plae and marked it at the bottom to divide into two sections.
2. Aseptically make a single-line streak inoculation of each test organism on the agar surface of its appropriately labeled section.
3. Incubate the plate in an inverted position at 37°C for 24 to 48 hours.

Observation:

Observe for zone of lipolysis, a clear zone surrounding the bacterial growth.

Result : In the case of positive lipid hydrolysis reaction, the loss of opacity occurs which is due to hydrolytic reaction, yielding soluble glycerol and fatty acids. In the case of negative test, no clear zone will be seen.

31. Oxidation Fermentation Test

Bacteria differ widely in their ability to metabolize carbohydrates and related compounds. For purposes of identification these differences can be demonstrated by four varieties of tests.

1. Test to distinguish between aerobic and anaerobic breakdown of carbohydrates.
2. Test to show the range of carbohydrates and related compounds that can be attacked
3. Test to show ability to utilize a particular substrate.
4. Test for specific breakdown products.

Carbohydrates are organic molecules that contain carbon, hydrogen and oxygen in the ratio $(CH_2O)_n$. These are of three types, i.e. monosaccharides (simple sugars containing 3-7 carbon atoms), disaccharides (containing two monosaccharide molecules) and polysaccharides (containing eight or more monosaccharide molecules). Chemical reactions, which release energy from the complex organic molecule decomposition, are referred to as catabolism. The capability of decomposition of carbohydrates is possessed by a large number of bacteria, fungi and yeasts. This microbial activity is of great significance in the carbon cycle in nature as well as in industries. Organisms use carbohydrate differently depending upon their enzyme complement. The pattern of fermentation is characteristic of certain species, genera, or groups of organisms and for this reason this property has been extensively used as a method for biochemical differentiation of microbes.

Extracellular enzymes as amylases and intracellular enzymes as maltase, lactase are utilized by the cell for metabolic degradation of carbohydrates. All the end products (glucose, galactose) of these reactions are used in glycolysis. Glucose after entering a cell can be catabolized either aerobically (biooxidations in which molecular oxygen (O_2) can serve as the final electron acceptor, i.e. oxidative metabolism) or anaerobically (bio-oxidations in which inorganic ions other than oxygen, e.g. NO_3^- or SO_4^{2-} can serve as final electron acceptor, i.e. fermentative metabolism) or both aerobic and anaerobic pathways and some organisms lack the ability to oxidize glucose by either. The metabolic end products of carbohydrate fermentation can be either organic acids (e.g. Lactic, acetic, formic) or organic acid and gas (e.g. H_2 or CO_2). Microorganisms which require organic carbon compounds as an energy source are called chemoorganotrophic.

The test used to distinguish between aerobic and anaerobic breakdown of a carbohydrate depends upon the use of a semi-solid tube medium containing the carbohydrate together with a pH indicators Bromothymol blue (BTB) or Bromocresol purple (BCP) [Hugh and Leifson's medium (OF medium)]. Two tubes are used, one open to the air and the other sealed with paraffin (to keep air out). Growth of the organisms in this medium is either by utilizing tryptone which results in an alkaline reaction (dark blue colour) or by utilizing glucose, which results in the production of acid (turning BTB to yellow). Those organisms, which produce acid in both closed and open tubes are described as fermentative while those which produce acid only in open tube are called oxidative.

Carbohydrates and related compounds may be altered by normal heat sterilization temperatures. They may also be altered when heated in presence of other ingredients of culture media, e.g. peptone, phosphate. It is thus recommended that the basal ingredients of test media should be heat sterilized before the carbohydrate or related compound is added. A solution of carbohydrate compound under test should be sterilized separately and the requisite amount subsequently added with aseptic precautions to the sterile basal medium. (filtration, steaming at 100°C, tyndallization).

Carbohydrates are particularly susceptible to heat in alkaline environment and if a carbohydrate solution is to be sterilized by heat, the prior addition of 1-2 drops of phosphoric acid will ensure that the solution is not alkaline.

Materials Required

(1) Two OF broth tubes
(2) Inoculating straight wire
(3) Bunsen burner
(4) Incubator
(5) Paraffin oil
(6) 24 hours broth culture of test organisms (*E.coli* and *Pseudomonas aeruginosa*).

Procedure

1. Inoculate duplicate tubes of OF medium with each test organism by stabbing with a straight wire.
2. Cover one of the tubes of each set promptly with a layer of sterile (molten petroleum jelly) liquid paraffin to a depth of about 1 cm.
3. Incubate at 37°C for 24-48 hours.

Observation and Results : Observe the tubes for the presence of the growth, colour of the medium and type of metabolism.

S.No.	Sample	Growth		Colour		Type of metabolism
		Open Tube	Plugged Tube	Open Tube	Plugged Tube	(Fermentative: oxidative or neither)
1.	*E. coli*					
2.	*Ps. aeruginosa*					

Precautions

1. The straight wire should not be stabbed up to the bottom of test tube.
2. Glucose should never be autoclaved along with the other constituents of the OF medium.
3. Sufficient amount of paraffin should be added to the inoculated tube to form a layer about 1cm in depth to create anaerobic condition.

Table 7.1 : Properties of *Escherichia coli* and some other enterobacteria.

	Escherichia coli	A-D Group	*Edwardsiella tarda*	*Klebsiella species*	*Enterobacter cloacae*	*Entero. aerogenes*	*Hafnia alvei*	*Serratia marcescens*	*Serratia liquefaciens*	*Citrobacter freundi*	*Citrobacter diversus*	*Proteus vulgaris*	*Proteus mirabilis*	*Proteus morgani*	*Proteus rettgeri*	*Provi. alcalifaciens*	*Providencia stuarti*	*Erwinia herbicola*
Motility	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Capsule	-	-	-	+	-	-	-	+/-	-	-	-	-	-	-	-	-	-	-
Clucose(gas)	+	-	+	+/-	+	+	+	+/-	+	+	+	+	+	+	-	+	-	-
Lactose (acid)	+	+/-	-	+/-	+	+	-	-	+/-	(+)/-	(+)/-	-	-	-	-	-	-	+/-
ONPG	+	+/-	-	+/-	+	+	+	+	+	+	+	-	-	-	-	-	-	+
Sucrose (acid)	+/-	+/-	-	+/-	+	+	+/-	+	+	+/-	+/-	+	(+)	-	(+)	(+)/-	(+)	+
Adonitol (acid)	-	-	-	+	+/-	+/-	-	+/-	-	-	+	-	-	-	+	+	-	-
Inositol (acid)	-	-	-	+	+/-	+	-	+/-	+	-	-	-	-	-	+	-	+	+/-
Mannitol (acid)	+	+	-	+	+	+	+	+	+	+	+	-	-	-	+	-	-	+
Indole	+	+	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	-
Methyl red	+	+	+	+/-	-	-	-	-	-	+	+	+	+	+	+	+	+	-
Voges-Proskauer	-	-	-	+/-	+	+	+	+	+/-	-	-	-	+/-	-	-	-	-	-
Citrate	-	-	-	+/-	+	+	+	+	+	+	+	+/-	+/-	-	+	+	+	+
Gelatin	-	-	-	-	(+)	+/-	-	+	+	-	-	+	+	-	-	-	-	+
Phenylalanine	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+
Urease	-	-	-	+/-	+/-	-	-	-	+/-	-	±	+	+	+	+	-	-	-
Hydrogen sulphide	-	-	+	-	-	-	+	-	-	±	-	+	+	-	-	-	-	-
Lysine decarboxylase	+	+	+	+/-	-	+	+	+	+	-	-	-	-	-	-	-	-	-
Ornithine decarboxylase	+/-	+/-	+	-	+	+	+	+	+	+/-	+	-	+	+	-	-	-	-

Note: +, result positive with at least 80% of strains; -, result negative with at least 80% of strains; +/-, some strains positive, some negative; (+), delayed positive result (e.g. 2-8 days) (+)/-, some strains delayed positive, some negtive, ±, weak reaction.

Section - VIII

Environmental Effect and Control of Microbial Growth

32. Effect of Temperature on Microbial Growth

The aims of this experiment are to reveal the diverse growth temperature requirements of bacteria and to determine whether optimum growth temperature is also the ideal temperature for other enzyme-regulated cell activities such as pigment production and carbohydrate fermentation.

Temperature is one of the most important factors influencing the activity of bacterial enzymes. Microbial growth is directly dependent on how temperature affects cellular enzymes. With increasing temperatures, enzyme activity increases until the three dimensional – configuration of these molecules is lost because of denaturation of their protein structure. On the other hand, as the temperature is lowered toward the freezing point, enzyme inactivation occurs and cellular metabolism gradually diminishes. At 0°C, biochemical reactions cease in most cells.

Bacteria, as a group of living organisms, are capable of growth within the temperature range of – 5°C to 80°C. Each species, however, requires a narrower range that is determined by the heat sensitivity of its enzyme systems. Specific temperature ranges consist of the following significant temperature points.

1. **Minimum growth temperature:** The lowest temperature at which growth will occur. Below this temperature, enzyme activity is inhibited and the cells are metabolically inactive so that growth is negligible or absent.
2. **Maximum growth temperature:** The highest temperature at which growth will occur. Above this temperature, most cell enzymes are destroyed and the organism dies. High temperatures cause coagulation and thus irreversibly denature thermolabile enzymes.

3. **Optimum growth temperature:** The temperature at which the rate of reproduction is most rapid. At the optimum temperature the enzymatic reaction progress at maximum rate.

On the basis of their temperature requirements, the bacteria can be classified into one of three major groups.

1. **Psychrophiles:** Bacterial species that will grow within temperature range of –5°C to 20°C. The distinguishing characteristic of all Psychrophiles is that they will grow between the temperature 0°C and 5°C.
2. **Mesophiles:** Bacterial species that will grow within a temperature range of 20°C.to 45°C. The distinguishing characteristics of all mesophiles are their ability to grow at human body temperature (37°C.) and their inability to grow at temperature above 45°C. Mesophiles are differentiated into two groups –
   a. Those whose optimum growth temperature is in the range of 20°C to 30°C are plant saprophytes
   b. Those whose optimum growth temperature is in the range of 35°C to 40°C are organisms that prefer to grow in the bodies of warm-blooded hosts.
3. **Thermophiles:** Bacterial species that will grow at 35°C and above. The two groups of thermophiles are:
   a. **Facultative Thermophiles:** Organism that will grow at 37°C with an optimum growth temperature of 45°C to 60°C.
   b. **Obligate Thermophiles:** Organisms that will grow only at temperatures above 50°C, with optimum growth temperatures above 60°C.

In this experiment, we will attempt to measure the effects of various temperatures on physiological reactions:

a. Pigment production
b. Growth rate
c. Carbohydrate fermentation

Serratia marcescens will be grown on nutrient agar slants at 25°C and 37°C to see which temperature favors the enzymatic reactions for pigment formation. This organism and *Escherichia coli* will also be grown in nutrient broth at various temperatures (4°C, 25°C, 37°C and 60°C) to determine, approximately, their optimum growth temperatures. *Saccharomyces cerevisiae* will be grown in sabouraud broth at different temperature to determine their optimum growth temperatures and also for carbohydrate fermentation.

The ideal temperature for specific enzymatic activities may not coincide with the optimum growth temperature for a given organism.

Materials Required:

Apparatus:

1. Bunsen burner,
2. Inoculating loop,
3. Refrigerator,
4. Incubators,
5. Sterile Pasteur pipette and
6. Glassware marking pencil.

Cultures: 24-48 hours nutrient broth cultures of *Escherichia coli, Serratia marcescens* and sabouraud broth culture of *Saccharomyces cerevisiae*.

Media: Nutrient agar slants, Nutrient broth tubes and sabouraud broth tubes containing inverted Durham tubes.

Procedure:

1. Label the nutrient agar slants, one as 25°C and the other 37°C and inoculate these slants with *S. marcescens* aseptically by means of a wire loop.
2. Label each nutrient broth tube with the name of the test organism (*E.coli, S. marcescens*) to be inoculated and the temperature of incubation 4°C, 25°C, 37°C and 60°C) and inoculate the tubes with appropriate (as labeled in tube) organisms aseptically.

3. Appropriately, label the four-subouraud broth tubes including the temperatures of incubation as indicated above and inoculate the tubes with *S. cerevisiae* aseptically.
4. Incubate all the tubes at appropriate (labeled) temperature for 24 hours.

Observation and Results:

Observe the culture for the presence of growth as well as pigment and gas formation in the tube:

a. Observe the *S. marcescens* growth on all the slants for the presence or absence of orange to deep red pigmentation and record the presence as (1+), (2+), (3+), and (-) for the absence of pigmentation.

b. Evaluate the amount of growth in *S. cerevisiae* cultures by noting the degree of developed turbidity and observe the *S. cerevisiae* cultures for the formation of gas in the Durham tube, which is indicative of carbohydrate fermentation and record the observations as follows: (1+) for minimal amount of gas; (2+) for a moderate amount of gas; (3+) for the large amount of gas; and (-) for the absence of gas.

c. Observe the amount of growth in *E.coli* and *S. marcescens* cultures broth tube by noting the developed turbidity and note the observation as : (1+) for scant growth ;(2+) for moderate growth; (3+) for abundant growth; and (-) for the absence of growth on the basis of turbidity.

	Ser. marcescens		*E. coil*	*Sac. cerevisiae*	
Temperature	Growth	Pigment	Growth	Growth	Gas
4°C (Refr. temp.)					
25°C(room temp.)					
37°C (Body temp.)					
60°C					
Results					

33. Effect of Hydrogen Ion Concentration (pH) on Microbial Growth

The main purpose of this experiment is to reveal the diverse hydrogen ion concentration requirements of microorganisms. Growth and survival of microorganisms are greatly influenced by the hydrogen ion concentration of the environment. The concentration of hydrogen ions, which is customarily designated by the term pH $(=\log 1/H+)$ limit the activity of enzymes with which an organism is able to synthesize new protoplast. Each species has the ability to grow within a specific pH range, which may be broad or limited, with the most rapid growth occurring within a narrow optimum range, These specific pH needs reflect the organism's adaptations to their natural environment. For example, enteric bacteria are capable of survival within a broad pH range, which is characteristic of their natural habitat, the digestive system. Bacterial blood parasites, on the otherhand, can tolerate only a narrow range, the pH of the circulatory system remains fairly constant at approximately 7.4. Although the specific pH range for bacteria is between 4 to 9, the optimum growth usually occurs between 6.5 to 7.5. But there are some notable exceptions, including the acetic acid bacteria and *Thiobacillus thioxidans* which grow in acidic pH below 4. Fungi, Molds and Yeasts prefer an acidic environment with optimum activities at a pH of 4 to 6.

Because a neutral or nearly neutral environment is generally advantageous to the growth of microorganisms, the pH of the culture medium is frequently adjusted to approximately pH 7. Metabolic activities of the microorganisms will result in the production of wastes, such as acids from carbohydrate degradation and alkali from protein break down and these will cause shifts in pH that can be detrimental to their growth. To retard this shift, chemical substances that act as buffers are frequently incorporated when the medium is prepared. A commonly used buffering system, involves the addition of equimolar concentration of K_2HPO_4, a salt of a weak base, and KH_2PO_4, a salt of weak acid. In a medium that has become acidic, the K_2HPO_4 absorbs excess H+ to form a weakly acidic salt and a potassium salt with the anion of the strong acid.

$$\underset{\text{Salt of a weak base}}{K_2HPO_4} + \underset{\text{strong acid}}{HCL} \longrightarrow \underset{\text{salt of a weak acid}}{KH_2PO_4} + \underset{\text{potassium chloride salt}}{KCL}$$

In a medium that has become alkaline, KH_2PO_4 releases H+to form water by combining with the excess OH^- and the remaining anionic portion of the weakly acidic salt combines with the cation of the alkali.

$$\underset{\text{Salt of a weak acid}}{KH_2PO_4} + \underset{\text{strong base}}{KOH} \longrightarrow \underset{\text{salt of a weak base}}{K_2HPO_4} + \underset{\text{water}}{H_2O}$$

In this experiment, you will test the degree of inhibition of organisms that results from media containing different concentration of hydrogen ions (pH).

Materials Required:

Apparatus:

1. Bunsen burner,
2. Sterile pipette,
3. Spectrophotometer and
4. Glassware marker pencil.

Cultures: 24 hours nutrient broth culture of *Escherichia coli, Staphylococcus aureus* and *Saccharomyces cerevisiae.*

Media : Nutrient broth of different pH range (Nutrient broth of pH 3,5,7, and 9.).

Procedure:

1. Label each of the nutrient broth tubes with pH of the medium (pH 3,5,7 and 9) and name of the organisms to be inoculated.
2. Using sterile pipette, inoculate a series of the tubes containing nutrient broth of different pH with *E.coli* by adding 0.1 ml of the culture to each tube.
3. Repeat step 2 for the inoculation of *S.aureus* and *S. cerevisiae* to various labeled tubes.
4. Incubate the *E.coli, A. faecalis* and *S. aureus* inoculated tubes for 24 to 48 hours at 37°C and the *S. cerevisiae* inoculated tubes for 48 to 72 hours at 25°C.

Observations and Results:

i. Using the spectrophotomter determine the optical density of all cultures tested and record the readings.

Test Organisms	Optical Density Readings			
	pH_3	pH_5	pH_7	pH_9
E.coli				
A. faecalis				
S. aureus				
S. cerevisiae				

ii. Record the pH range and optimum pH of each organism.

Precuation:

1. The pH of the medium should be accurate. The pH is adjusted with 1N sodium hydroxide or 1N hydrochloric acid.

34. Effect of Atmospheric Oxygen on Microbial Growth

Microorganisms exhibit great diversity in their ability to use free oxygen (O_2), for cellular respiration. These variations in O_2 requirements reflect the differences in bio-oxidative enzyme systems present in the various species. On the basis of O_2 requirements, organisms can be grouped as:

1. **Aerobes:** These organisms require the presence of atmospheric oxygen for their growth. Their enzyme system necessitates use of O_2 as the final hydrogen (electron) acceptor in the complete oxidative degradation of high- energy molecules such as glucose. Aerobes cannot exist without O_2.
2. **Microaerophiles:** They require limited amounts of atmospheric oxygen for their growth. Oxygen in excess of the required amount appears to block the activities of their oxidative enzymes and results in death.

3. **Obligate anaerobes:** They require the absence of free oxygen for their growth because their oxidative enzyme system requires the presence of molecules other than O_2 to act as the final hydrogen (electron) acceptor. In these organisms, as in aerobes, the presence of atmospheric oxygen results in the formation of toxic metabolic end products, such as superoxide, peroxide, O_2^- a free radical of oxygen. As these organisms lack the enzymes superoxide dismutase and catalase, the superoxide, so produced, is not degraded to water and oxygen.

$$2O_2+2H^+ \xrightarrow{\text{Superoxide dismutase}} H_2O_2+O_2$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O+O_2$$

Therefore, in the absence of these enzymes, small amount of atmospheric oxygen are lethal and justifiably these organisms are called obligate anaerobes.

4. **Aerotolerant anaerobes:** These are fermentative organisms, and therefore they do not use O_2 as a final electron acceptor. As they produce catalase and/or superoxide dismutase enzyme, they are not killed by the presence of O_2. Hence, these organisms are anaerobes that are termed aerotolerant.
5. **Facultative anaerobes:** These organisms can grow in the presence or absence of free oxygen. They preferentially use oxygen for aerobic respiration. However in an oxygen poor environment, cellular respiration may occur anaerobically, utilizing nitrates (NO_3^-) or sulfates (SO_4^{2-}) as final hydrogen acceptors, or via a fermentative pathway.

The oxygen need of microorganisms can be determined by noting their growth distributions following a shake–tube inoculation. This procedure requires introduction of the inoculum into a molten agar medium, shaking of the test tube to disperse the microorganisms throughout the agar and rapid solidification of the medium to ensure that the cells remain dispersed. After incubation, the growth distribution indicates the organism's oxygen requirements.

Materials Required:

Apparatus:

1. Bunsen burner,
2. Water bath,
3. Thermometer,
4. Sterile Pasteur pipettes,
5. Glassware marking pencil.

Culture: 24 to 48 hours nutrient broth cultures of *Staphylococcus aureus*, and *Enterococcus faecalis*, 48 to 72 hours sabouraud broth cultures of *Saccharomyces cerevisiae*, *Aspergillus niger* and 48 hours thioglycollate broth culture of *Clostridium sporogenes*.

Media : Brain heart infusion agar deep tubes.

Procedure:

1. Liquefy the sterile infusion agar by boiling in a water bath at 100°C.
2. Cool molten agar to 45°C .
3. Using sterile technique, inoculate each test organism by introducing two drops of culture from a sterile pipette into the appropriately labeled tubes of molten agar.
4. Vigorously shake the freshly inoculated molten agar to distribute the organisms.
5. Place the inoculated test tubes in the ice water bath to solidify the medium.
6. Incubate the tubes of *Staphylococcus aureus*, *Enterococcus faecalis* and *Clostridium sporogenes* at 37°C for 24 to 48 hours. And the tubes of *A. niger* and *S. cerevisiae* at 25°C for 48 to 72 hours.

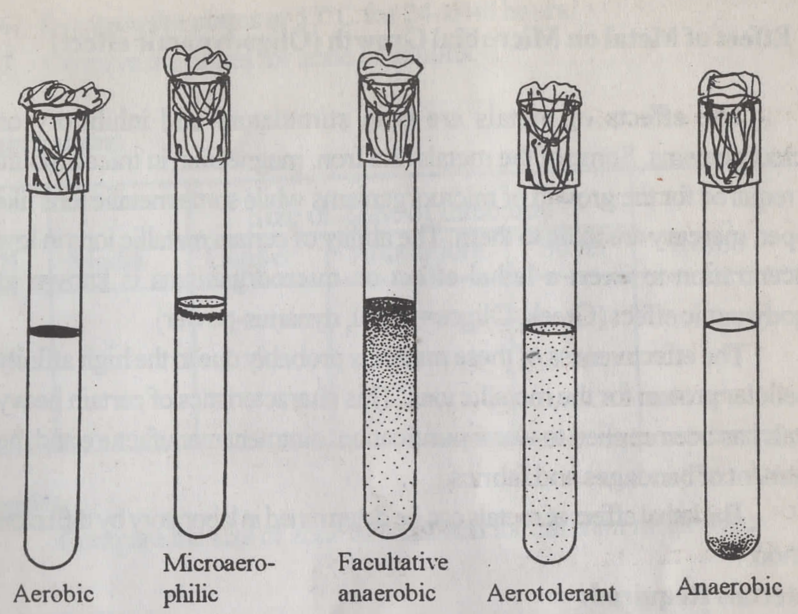


Figure 8.1 : Growth of Organisms based on O_2 Requirement

Observations and Results:

After proper incubation, observe each of test cultures for the distribution of growth in each tube.

1. Aerobes: Exhibit surface growth
2. Microaerophlies: Exhibit growth in a zone slightly below the surface
3. Facultative anaerobes: Exhibit growth throughout the medium but it may be grown densely at the bottom of the medium.
4. Anaerobes: growth is limited to the bottom of the deep tube.
5. Aerotolerant: Exhibits the growth uniformly throughout the medium.

Observations:

S. N.	Organisms	Distribution of Growth	Results

35. Effect of Metal on Microbial Growth (Oligodynamic effect)

The effects of metals are both stimulatory and inhibitory on microorganisms. Some of the metals like Iron, magnesium in trace amount are required for the growth of microorganisms while some metalic ions like copper, mercury are toxic to them. The ability of certain metallic ions in low concentration to exert a lethal effect on microorganisms is known as oligodynamic effect (Greek, Oligos= small, dynamis-power).

The effectiveness of these matals is probably due to the high affinity of cellular protein for the metallic ions. This characteristics of certain heavy metals has been applied to water purification, ointment manufacture and the treatment of bandages and fabrics.

The lethal effect of metals can be determined in laboratory by diffusion method.

Materials Required:

1. Bunsen burner
2. Foceps
3. Sterilized cotton swab
4. Nutrient agar plates
5. Alcohol
6. Different metal coins (copper, aluminium, silver)
7. Broth culture of *E.coli, S. aureus*.

Procedure:

1. Take two dry nutrient agar plates and label these with the name of each test organisms.
2. Inoculate the plates by swabing the respective test organisms from the culture broth tube with the help of sterile cotton swab.
3. Sterilize the coins by dipping first in alcohol and then flaming in burner with the help of forcep.
4. After cooling the coin, place it on the agar surface already swabbed with test organism. Place the coins evenly spaced.
5. Keep the plates diffusion for few minutes at room temperature for diffusion of metallic ions.

6. Incubate the plates at 37°C for 24 to 48 hours.
7. Observe the plates for zone of inhibition.

Observations

S.N.	Media	Size of Zone of Inhibition			Result
		Copper	Aluminium	Silver	

Results

Compare the size of zone of inhibition for different metal coins.

Precautions:

1. It is preferable that the coins should be of equal size.
2. While placing the coins over agar surface, slightly press the coins with forcep so that the coins remain attached to the agar surface.

36. Antimicrobial Subsceptibility Test (Kirby-Bauer Method)

The main aim of this experiment is to evaluate the antimicrobial activity of antimicrobics. There are many antibiotics and chemotherapeutic agents available in the market and they vary in their scope of antimicrobial activity. Some of them have a limited spectrum of activity, being effective against only one group of microorganisms. Others exhibit broad-spectrum activity against a range of microorganisms. The drug susceptibilities of many pathogenic microorganisms are known, but it is sometimes necessary to test several agents to determine the drug of choice. To determine a therapeutic drug of choice, one must know its mode of action, possible adverse side effects in the host, and the scope of its antimicrobial activity. The specific mechanism of action varies among different drugs and the short-term or long-term use of many drugs can produce systemic side effects in the host.

Table 8.1 : Prototypic Antibiotics

Antibiotic	Mode of Action	Possible Side Effects
Penicillin	Prevents transpeptidation of the N-acetylmuramic acids, producing a weakened peptidoglycan structure.	Penicillin resistance; sensitivity (allergic reaction)
Streptomycin	Has an affinity for bacterial ribosomes, causing misreading of codons on mRNA, thereby interfering with protein synthesis	May produce damage to auditory nerve, causing deafness
Chloramphenicol	Has an affinity for bacterial ribosomes, preventing peptide bond formation between amino acids during protein synthesis	May cause aplastic anemia, which is fatal because of destruction of RBC-forming and WBC-forming tissues
Tetracyclines	Have an affinity for bacterial ribosomes; prevent hydrogen bonding between the anticodon on the tRNA-amino acid complex and the codon on mRNA during protein synthesis.	Permanent discoloration of teeth in young children
Bacitracin	Inhibits cell-wall synthesis	Nephrotoxic if taken internally; used for topical application only
Polymyxin	Destruction of cell membrane	Toxic if taken internally; used for topical application only.

A standardized filter-paper disc-agar diffusion procedure, known as the Kirby-Bauer Method, is frequently used to determine the drug susceptibility of microorganisms isolated from different infectious processes. This method allows for the rapid determination of the efficacy of a drug by measuring the diameter of the zone of inhibition that results from diffusion of the agent into the medium surrounding the disc.

The susceptibility of an organism to a drug is determined by the size of the zone of inhibition, which itself is dependent on the following variables.

1. The ability and rate of diffusion of the antimicrobics into the medium and its interaction with the test organism.
2. The number of organisms inoculated.
3. The growth rate of the organisms.
4. The degree of susceptibility of the organism to the antimicrobics.

A measurement of the diameter of the zone of inhibition in millimeters is made and its size is compared to that contained in a standardized chart, (zone size interpretive chart). Based on this comparison, the test organism is determined to be resistant, intermediate, or susceptible to the antimicrobics.

Materials Required:

Apparatus:

1. Antimicrobial sensitivity discs (Antibiotic discs),
2. Forceps,
3. Bunsen burner,
4. Sterile cotton swab,
5. Glassware marking pencil and
6. Millimeter ruler.

Media: Mueller Hinton agar plates.
McFarland standards.

Cultures: 0.85% saline suspensions of *E. coli, Staphylococcus aureus, Pseudomonas aeruginosa*.

Procedure :

1. Place agar plates right side up in an incubator at 37°C for 10-20 minutes with the covers adjusted so that the plates are slightly opened.
2. Label each plates with the name of test organism to be inoculated.
3. Using aseptic technique, inoculate all agar plates with their respective test organisms as follows:
    a. Dip a sterile cotton swab into a well mixed saline test culture (the density of half of the McFarland standard 1) and remove excess inoculum by pressing the saturated swab against the inner wall of the culture tube.
    b. Using the swab, streak the entire agar surface horizontally and vertically to ensure a heavy growth over the entire surface.
4. Allow all culture plates to dry for about 5 minutes.
5. Using aseptic technique apply the antibiotic discs on the agar surface by using sterile forceps. Keep each disc at least 15 mm from edge of plate.
6. Gently press each disc down with the sterile forceps to ensure that the discs adhere to the surface of the agar.

Table 8.2 : Zone Diameter Interpretive Standards for Organisms other than Haemophilus and Neisseria gonorrhoeae

Antimicrobial Agent	Disk Content	Zone Diameter, Nearest Whole mm Resistant	Intermediate	Susceptible
Ampicillin				
when testing gram-negative enteric organisms	10 µg	≤13	-	≥17
when testing staphylococci	10 µg	≤28	-	≥29
when testing enterococci	10 µg	≤16	-	–
when testing non-enterococcal streptococci	10 µg	≤21	-	≥30
when testing Listeria monocytogenes	10 µg	≤19	-	≥20
Carbenicillin				
when testing Pseudomonas	100 µg	≤13	-	≥17
when testing other gram-negative organisms	100 µg	≤19	-	≥23
Chloramphenicol	30 µg	≤12	13-17	≥18
Erythromycin	15 µg	≤13	14-22	≥23
Gentamicin	10 µg	≤12	13-14	≥15
Kanamycin	30 µg	≤13	124-17	≥18
Methicillin when testing staphylococci	5 µg	≤9	10-13	≥14
Novobiocin	30 µg	≤17	18-21	≥22
Penicillin-G				
when testing staphylococci	10 µg	≤28	-	≥29
when testing enterococci	10 µg	≤14	-	–
when testing L. monocytogenes	10 µg	≤19	-	≥20
when testing non-enterococcal streptococci	10 µg	≤19	-	≥28
Rifampin	5 µg	≤16	17-19	≥20
Sreptomycin	10 µg	≤11	12-14	≥15
Tetracycline	30 µg	≤14	15-18	≥19
Trimethoprims/sulfamethoxazole	1.25/23.75 µg	≤10	-	≥16
Vancomycin				
when testing enterococci	30 µg	≤9	10-11	–
when testing other gram-positives	30 µg	≤9	10-11	≥12
Urinary Tract Specific Antimicrobial Agents				
Sulfonamides	250 or 300 µg	≤12	-	≥17
Trimethoprim	5 µg	≤10	-	≥16

7. Incubate all the plate culture in an inverted position for 24 to 48 hours at 37°C.

Observations:

1. Examine all the plates for presence or absence of a zone of inhibition surrounding each disc.
2. Carefully measure each zone of inhibition by using a ruler graduated in millimeters and record the results.
3. Compare the results with the standard chart and indicate susceptibility of each test organism to the chemotherapeutic agent as resistant (R), intermediate (I) or sensitive (S).

S.No	Chemotherapeutic agents	*E.coli*		*S. aureus*		*Ps. aeruginosa*	
		Zone size	Susceptibility				
	Penicillin Gentamycin						

Precautions:

1. While pressing the discs, do not press the discs into the agar.
2. Ignore faint growth or tiny colonies that can be detected by very close scruting.
3. Ignore the "swarming" characteristic of *proteus*, measuring to the margin of heavy growth.
4. Accuracy of the test depends on the disc potency, proper inoculum, functional pretested medium plates (nature of Medium and its depth), inoculation technique, incubation temperature and time.

Section - IX

The Fungi

The fungi, a term that includes both yeasts and molds, differ significantly from the bacteria. The fungi are eukaryotic higher protists and contain a definite nucleus with a surrounding nuclear membrane; their cell wall is primarily composed of glucose and mannose polymers (chitin); their cell membrane contains sterols; and the organisms are resistant to antibacterial antibiotics. They show marked susceptibility to polyene antifungal agents and all reproduce asexually; however, some reproduce sexually as well.

The unicellular yeasts and multicellular molds can be easily distinguished on the basis of macroscopic appearance of the colonies formed. The yeasts produce moist, creamy, opaque, or pasty colonies on culture media, whereas the filamentous molds produce fluffy, cottony, woolly or powdery colonies. Certain slow growing pathogenic fungi are dimorphic and produce a mold form at 25°-30°C and a yeast or spherule form at 35°-37°C under certain circumstances.

The identification of the yeasts requires not only recognition of certain microscopic features, but also the use of biochemical tests to provide a definite species identification. The definitive identification of the molds requires recognition of the characteristic microscopic features of the organism.

The yeasts usually reproduce by budding; their microscopic morphological features appear similar for different genera and are not particularly helpful in their separation. The basic structural units of molds are tube like projections known as **hyphae**. As the hyphae grow, they become interwined to form a loose network called the **mycelium**, which penetrates the substrate from which it obtains the necessary nutrients for growth. The nutrient absorbing and water exchanging portion of the fungi is called the **vegetative mycelium**. The portion projecting above the substrate surface is known as the **aerial mycelium**, aerial mycelia often give rise to fruiting bodies from which the asexual spores are borne. Recognition of certain types of vegetative hyphae is helpful in the identification of the organism. eg. dermatophytes often produce several types of hyphae, including antler hyphae that are curved, free branching and antlerlike in appearance. Racquet hyphae are often found that are enlarged, club shaped hyphae with the smaller end attached to the large end of an adjacent club shaped hyphal strand. In addition, certain dermatophytes produce spiral hyphae that are coiled or cork screw like turns seen within the hyphal strand. These structures are not characteristic for certain group of organisms, however, they are usually found only in the dermatophytes.

The features by which most fungi are identified are related to the mode of sporulation and the morphology and arrangement of the spores produced. Fungi may reproduce sexually, asexually or by both means. Sexual reproduction is associated with the production of specialized structures that result in nuclear fission and production of specialized spores. Some species of fungi produce sexual spores in a large saclike structure called an **ascocarp**. This, in turn, contains smaller sacs called asci, each of which contains 4 or 8 ascospores.

In true yeasts, sexual reproduction results in the formation of ascospores, the yeast cell serving as an ascus. The formation of ascospores follows conjugation of two cells but some may produce ascospores without conjugation. The usual no of spores per ascus and the appearance of the ascospores are characteristic of a kind of yeast. The ascospores may vary in colour, smoothness/roughness of walls and their shape (round, oval, bean or sickle shaped, Saturn or hat shaped, hemispherical, angular, fusiform or needle shaped). False yeasts don't produce ascospores or other sexual spores.

Basidiospores are single celled spores borne on club shaped structure called **basidium**. Nuclear fission and meiosis take place in basidium. The spores are then formed exogenously at tips of special outgrowths called **sterigmata**. The shape of basidium is different for different species. Usually there are 4 projections (sterigmata) and 4 spores are produced, one at the end of each sterigma. If meiosis is followed by mitosis, 8 basidial nuclei may be formed but rarely do all develop into basidiospores. **Zygospores** are large, thick walled spores formed by the union of tips of two sexually compatible hyphae often appearing similar and may come from same or different mycelia i.e. union of two gametangia. **Oospores** are formed when male antheridium fuses with oospheres (eggs) within large female oogonium i.e. gamete gametangeal copulation. There may be one or more eggs in each oogonium.

There is a consensus that perhaps all fungi possess a sexual form; however, this form has not been observed on artificial culture media for most of the clinically important species. Asexual sporulation is the type of sporulation seen in most fungi encountered in a laboratory. The type of spores, their morphology and arrangement are important criteria for establishing the definite identification of an organism.

The simplest type of sporulation is the development of the spore directly from the vegetative hyphae. **Arthrospores** (arthroconidia) are formed directly from the hyphae or oidia by fragmentation through the points of septation. When mature, they appear as square, rectangular, or barrel shaped, thick walled cells. These result from the simple fragmentation of the hyphae into spores, which are easily dislodged and disseminated into the environment e.g. *Geotrichum*. **Chlamydospores** (chlamydoconidia) are round, thick walled resistant spores formed directly from the differentiation of the hyphae in which there is a concentration of protoplasm and nuclear material. These spores appear to be resistant resting spores produced by the rounding up and enlargement of the terminal cells of the hyphae. **Blastospores** (blastoconidia or budding spores) eg. *Trichophyton, Epidermophyton, Microsporum*, most often found in yeasts, are cells produced by budding with the daughter cells being pinched off from portions of the mother cell through a constricted area. In some species of yeasts, eg. *Candida*, blastoconidia may elongate and remain attached and form structures called **pseudohyphae**.

Conidia are asexual spores produced singly or in groups by specialized vegetative hyphal strands called **conidiophores**. In some instances, conidia are freed from their point of attachment by pinching off or abstrictions. Some conidiophores terminate in a swollen structure called a **vesicle**. From the surface of the vesicle are formed secondary small flask shaped phialides, which in turn give rise to long chains of conidia. This type of fruiting structure is characteristic of *Aspergillus*. A single, slender, tubular conidiophore (phialide) that supports a cluster of conidia, held together as a gelatinous mass, is charactertic of certain fungi, including *Acremonium*. In other instances, conidiophores branch into a structure known as a **penicillus** in which each branch terminates in secondary branches (metulae) and phialides from which chains of conidia are borne. Species of *Penicillium* and *Paecilomyces* are representative of these type of sporulation. Conidia may be of two forms microconidia are small, unicellular, round, elliptical, or pyriform in shape and macrocondia are large, usually multiseptate, and club or spindle shaped. Microconidia may be borne directly on the side of the hyphal strand or at the end of long or short conidiophores. Macroconidia are usually borne on a short to long conidiophore and may be smooth walled or rough walled. Microconidia and macroconidia are seen in many fungal spacies eg. *Alternaria* and are not specific, except when they are used to differentiate the different genera of dermatophytes. One group of fungi, the zygomycetes, produces structures different from the ones previously described. The hyphae are sparsely septate, and asexual spores are produced in a large sac like structure called **sporangium**, which is borne on the tip of a supporting structure (sporangiophore). Sporulation takes place by progressive cleavage during maturation within the sporangium, the sporangiosposes are produced and released by the rupture of the sporangial wall. This type of sporulation is characteristic of zygomycetes as *Mucor* and *Rhizopus*. Aplanospores are non-motile sporangiospores and zoospores are motile (flagellated) sporangiospores.

Although a single fungus may produce asexual and sexual spores by several methods at different times and under different conditions, the spores are sufficiently constant in their structures and in the method by which they are produced to be used in identification and classification.

Criteria used in differentiation and Identification

Molds

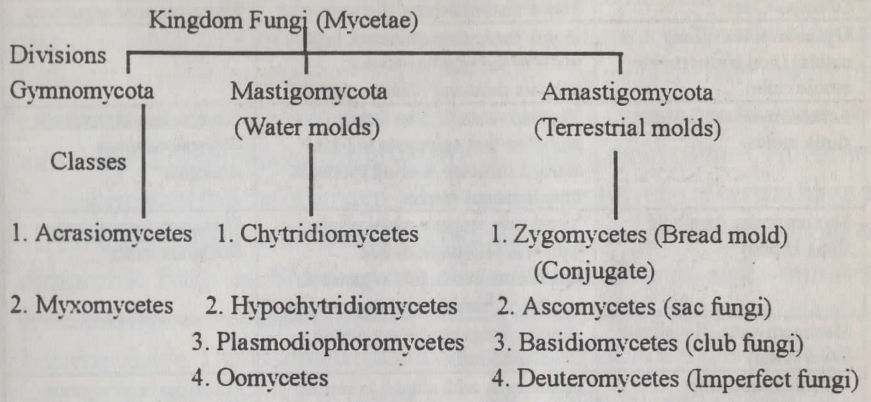
1. Hyphae – septate or non septate
2. Mycelium – clear or dark (smokey)
3. Mycelium – coloured or colourless
4. Sexual spores and types.
5. Asexual spores and types.
6. Characteristics of spore head.
    a. Sporangia – size, colour, shape, location

   b. Spore heads bearing conidia – arrangement of conidia (chains, budding, single or masses), shape and arrangement of sterigmata or phialides).
7. Appearance of sporangiophores or condidophores – simple or branched (type of branching), size and shape of columella at tip of sporangiophore, conidiophores – single or in bundles.
8. Microscopic appearance of asexual spores especially of conidia, size, shape, colour, smooth or rough; 1,2 or many celled.
9. Presence of special structures (or spores) e.g. stolens, rhizoids, foot cells, apophysis, chlamydospores, sclerotia, etc.

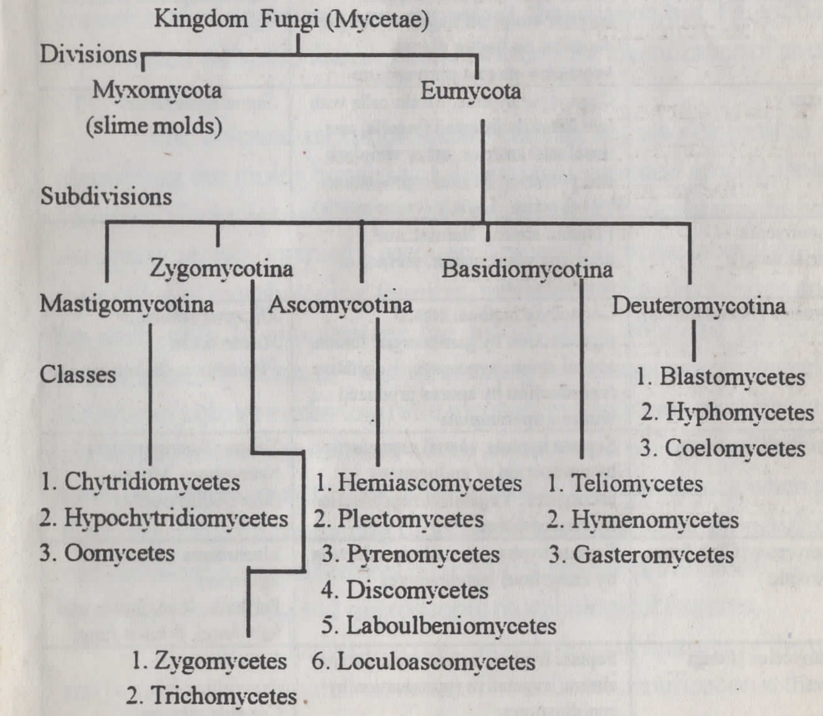
Yeasts

1. Whether ascospores are formed or not.
2. If they are spore forming.
   a. Method of ascospore production
   i. Without conjugation – conjugation of ascospores.
   ii. Isogamic conjugation – conjugation of small daughter cells
   iii. Heterogamic conjugation.
   b. Appearance of ascospores – shape, size, colour
   c. Number of ascospores per ascus.
3. Appearance of vegetative cells – shape, size, colour.
4. Method of asexual reproduction.
5. Production of mycelium, pseudomycelium or no mycelium.
6. Film yeast or fermentative
7. Colour of macroscopic growth.
   a. N_2 and C sources
   b. Vitamin Requirements
   c. Lipolysis, urease activity, acid production

Figure 9.1 : Classification and major properties of fungi.


Kingdom Fungi (Mycetae)
Divisions
Gymnomycota
Mastigomycota (Water molds)
Amastigomycota (Terrestrial molds)
Classes
1. Acrasiomycetes
2. Myxomycetes
1. Chytridiomycetes
2. Hypochytridiomycetes
3. Plasmodiophoromycetes
4. Oomycetes
1. Zygomycetes (Bread mold) (Conjugate)
2. Ascomycetes (sac fungi)
3. Basidiomycetes (club fungi)
4. Deuteromycetes (Imperfect fungi)

(Modified after Alexopoulos, 1979)


Kingdom Fungi (Mycetae)
Divisions
Myxomycota (slime molds)
Eumycota
Subdivisions
Mastigomycotina
Zygomycotina
Ascomycotina
Basidiomycotina
Deuteromycotina
Classes
1. Chytridiomycetes
2. Hypochytridiomycetes
3. Oomycetes
1. Zygomycetes
2. Trichomycetes
1. Hemiascomycetes
2. Plectomycetes
3. Pyrenomycetes
4. Discomycetes
5. Laboulbeniomycetes
6. Loculoascomycetes
1. Teliomycetes
2. Hymenomycetes
3. Gasteromycetes
1. Blastomycetes
2. Hyphomycetes
3. Coelomycetes

(Based on Ainsworth, 1973)

Table 9.1 : Classification and Characteristics of fungi.

Division/Class	Major distinguishing characteristics	Representative organisms
Gymnomycota (Slime molds) (non phototrophic motile cells)	Ingest particulate nutrients, lack cell walls in the vegetative stage, habitats decaying plant matter, soil	-
Acrasiomycetes (Cellular slime molds)	Vegetative stage: free living amoebae that aggregate to form stalked sorocarp bearing spores in mucllagenous matrix.	*Dityostelium discoideum* *Polysphondelium vioaceum*
Myxomycetes (acellular slime molds)	Vegetative stage: a multicellular, wall less plasmodium that transforms into highly organized sporangia bearing sporangcospores	*Physarum polycephalum* *Didimium iridis*
Mastigomycota (flagellated lower fungi)	Aquatic fungi producing motile flagellated cells.	
- Chytridiomycetes	molde cells with single, posterior, whiplash flagellum	*Allomyces macrogymus*
Hypochytridiomycetes	motile cells with single, anterior, tinsel flagellum	*Rhizidiomyces arbuscula* *Hypochytrium catenoides*
Plasmodiophoromycetes	motile cells with two unequal, anterior whiplash flagella. Obligate parasites on higher plants, vegetative stage a plasmodium	*Plasmodiophora brassica*
Oomycetes	Coenocyte hyphae. Motle cells with two laterally inserted flagella, one tinsel and anterior, other whiplash and posterior. Sexual reproduction by oospcres. Aquatic (water molds)	*Saprolegina ferax*
Amastigomycota (Terrestrial fungi)	Flagella absent. Habitat: soil, decaying plant matter, surface of animal bodies.	
Zygomycetes (Bread molds)	Coenocyte hyphae, sexual reproduction by gametangial fusion, sexual spore: zygospore. Vegetative reproduction by spores produced within a sporangium	*Rhizopus stolonifer* *Mucor rouxii* *Phycomyces blakesleanus*
Ascomycetes (Sac fungi)	Septate hyphae, sexual reproduction by production of endogenous ascospores. Vegetative reproduction by condiospores	Yeasts (*Saccharomyces*) *Neurospora*, Morels (*Morchella*) truffles
Basidiomycetes (Club fungi or mushrooms)	Septate hyphae, sexual reproduction by exogenous basidiospores	Mushrooms (*Amanita, Agaricus*) Puffballs, Rust, Smuts and jelly fungi, bracket fungi
Deuteromycetes (Fungi imperfecti)	Septate hyphae, sexual reproduction absent, vegetative reproduction by conidiospores	*Penicillium sps.* *Aspergillus sps.* *Candida albicans* *Alternaria tenius*

Identification of Fungi

The identification of fungi is made using a combination of I) growth rate 2) colonial morphological features, and 3) microscopic morphological features. In most instances, the latter provides the most definite means for identification. The determination of growth rate of a culture can be one of the most helpful observations made when examining a mold culture. However, this information may be of limited value since the growth rate of certain fungi is variable, depending on the amount of inoculum. In general, the growth rate of dimorphic fungi as *Blastomyces dermatitidis, Coccidioides immitis, Histoplasma capsulatum* is slow, 1-4 weeks are required before colonies become visible. This is somewhat uncommon circumstance and is encountered only when large number of organisms are present on culture medium. In contrast, colonies of the zygomycetes appear within 24 hours whereas the other hyaline and dermatiaceous fungi often exhibit growth in 1-5 days. The growth rate of organism is an important observation but it must be used in combination with other features before the definite identification of an organism can be made.

The colonial morphological features are also of limited value in identifying the molds because of the natural variation among isolates and variation of colonies on different culture media. Although it may be possible to recognize certain organisms recovered repeatedly in the laboratory, based on their colonial morphological features, this is an unreliable criterion and should be used only to supplement the information obtained by microscope examination of the culture. When examining the colonial morphological features, the type of culture media used and incubation conditions must be considered. e.g. *Histoplasma capsulatum* which appears as a white to tan fluffy mold on brain heart infusion agar, may appear yeast like in appearance when grown on the same medium containing blood enrichment. It is recommended that the colonial morphological features be used to supplement the information obtained from the growth rate and microscopic morphological features.

In general, the microscopic morphological features of the molds are stable and exhibit minimal variation. The definite identification is based on the characteristic shape, method of production, and arrangement of spores; however, the size of the hyphae also provides helpful information. The large ribbon-like hyphae of the zygomycetes are easily recognized, whereas small hyphae (1-2 µm in size) may suggest the presence of one of the dimorphic fungi.

The fungi may be prepared for microscopic observation using several techniques. The procedure traditionally used by most laboratories is the wet mount examination. The wet mount can be prepared easily and quickly and often is sufficient to identify many of the fungi.

The easiest, most economical, and the most suitable method for the microscopic identification of fungi is the adhesive tape preparation. This method is recommended for routine use in clinical laboratories.

Some laboratories prefer to use the micro slide culture for making the microscopic identification of an organism. This method might appear to be most suitable since it allows once to observe microscopically the fungus growing directly underneath the cover ship. Microscopic features should be easily discerned, structures should be intact, and a large number of representative areas available for observation.

37. Culture and Isolation of Fungi

Fungi can be cultured/isolated in laboratory under favourable condition. This requires the media as Potato Dextrose Agar, Saboraud Dextrose agar, or Czapek dox agar. The media plate is inoculated with the specimen (e.g. by exposure, imprint, streaking, spot inoculation, etc.) and incubated usually at room temperature (25°C to 28°C) for 2-3 days. These media contain high sugar concentration and low pH that favour the good growth of fungi. Lactophenol blue, cotton blue, Aniline blue are the commonly used fungal stains.

Wet Mount

- With a wire bent at a 90°C angle, cut out a small portion of an isolated colony. The portion should be removed from a point intermediate between the center and the periphery. The portion removed should contain a small amount of the supporting agar.
- Place the portion of culture onto a slide to which has been added a drop of lactophenol cotton or aniline blue.

- Place a cover slip into position and apply gentle pressure to disperse the growth and agar.
- Examine microscopically.

The main drawback of wet mount is that the characteristic arrangement of spores is disrupted when pressure is applied to the cover slip. And when their arrangement cannot be determined, it is not adequate to make definite identification.

Adhesive (Scotch) tape preparation

- Touch the adhesive side of a small length of transparent cellophane tape to the surface of the colony.
- Adhere the tape to the surface of a microscopic slide to which has been added a drop of lactophenol cotton or aniline blue.
- Observe microscopically for the characteristic shape and arrangement of the spores.

The scotch tape preparation allows one to observe the organism microscopically approximately the way it sporulates in culture. The spores are usually intact, and the microscopic identification of an organism can be made with ease. If the tape is not firmly pressed enough to the colonies' surface, the sample may not be adequate for an identification. In instances when spores are not observed, a wet mount should be made as a backup step.

Micro Slide Culture Technique

The slide culture technique is used to observe morphological characteristic of molds without disturbing the arrangement of spores and hyphae. Other important application of this technique lies in studying the conidial ontogeny over a period of time in a given area of the preparation.

- Cut a small block of suitable agar medium that has been previously poured into a culture dish to a depth of approximately 2 mm. The block may be cut using a sterile scalpel, blade or with a sterile test tube (without lip-to produce a round block).

- Place a sterile slide onto the surface of a culture dish containing sterile 2% agar. (Alternatively, place a round piece of filter paper or paper towel into a sterile culture dish, add two applicator sticks, and position the microscope slide on top).
- Add the agar block to the surface of the sterile microscopic slide.
- With a right angle wire, inoculate the four quadrants of the agar plug with the organisms.
- Apply a sterile cover slip onto the surface of the agar plug.
- If the filter paper applicator stick method is used, add a small amount of sterile water to the bottom of the culture dish. Replace the lid of the culture and allow it to incubate at 30°C.
- After incubation, remove the cover slip and place it on a microscope slide containing a drop of lactophenol cotton or aniline blue.
- Observe microscopically for the characteristic shape and arrangement of spores.
- The remaining agar block may be used later if it is allowed to incubate further. The agar plug is removed and discarded, and a drop of lactophenol cotton or aniline blue is placed on the area of growth and a cover slip is positioned onto place. It is likely to make two cultures on the same slide so that if characteristic microscopic features are not observed on examination of the first culture, the second will be available after an additional incubation period.

Although this method is ideal for making a definite identification of an organism, it is the least practical of all the methods described. It should be limited to those instances in which identification cannot be made on the basis of an adhesive tape preparation or wet mount. It is not likely to make slide cultures of slowly growing organisms suspected to be dimorphic pathogens. Micro slide cultures must be observed only after a cover slip has been removed from the agar plug and not while it is in position on top of the agar plug. The latter method of observation is highly dangerous and is not to be used in the clinical laboratory.

Preparation of Semi Permanent Mounts

I. From Cover Slip

- Gently lift up the cover slip from the surface of agar with forceps.
- Put a drop of 95% alcohol on fungus slide of the cover slip.
- Drain off the alcohol.
- Place a drop of lactophenol cotton blue on a clean slide and put the cover slip over it with the fungus face facing down.
- Examine the slide microscopically.

II. From Slide

- Gently remove an agar block from the slide using a clean blade.
- Place the agar block in the disinfectant solution.
- Put a drop of 95% alcohol on fungus growth on slide.
- Drain off the alcohol.
- Place a drop of lactophenol cotton blue on fungal growth.
- Place a clean cover slip.

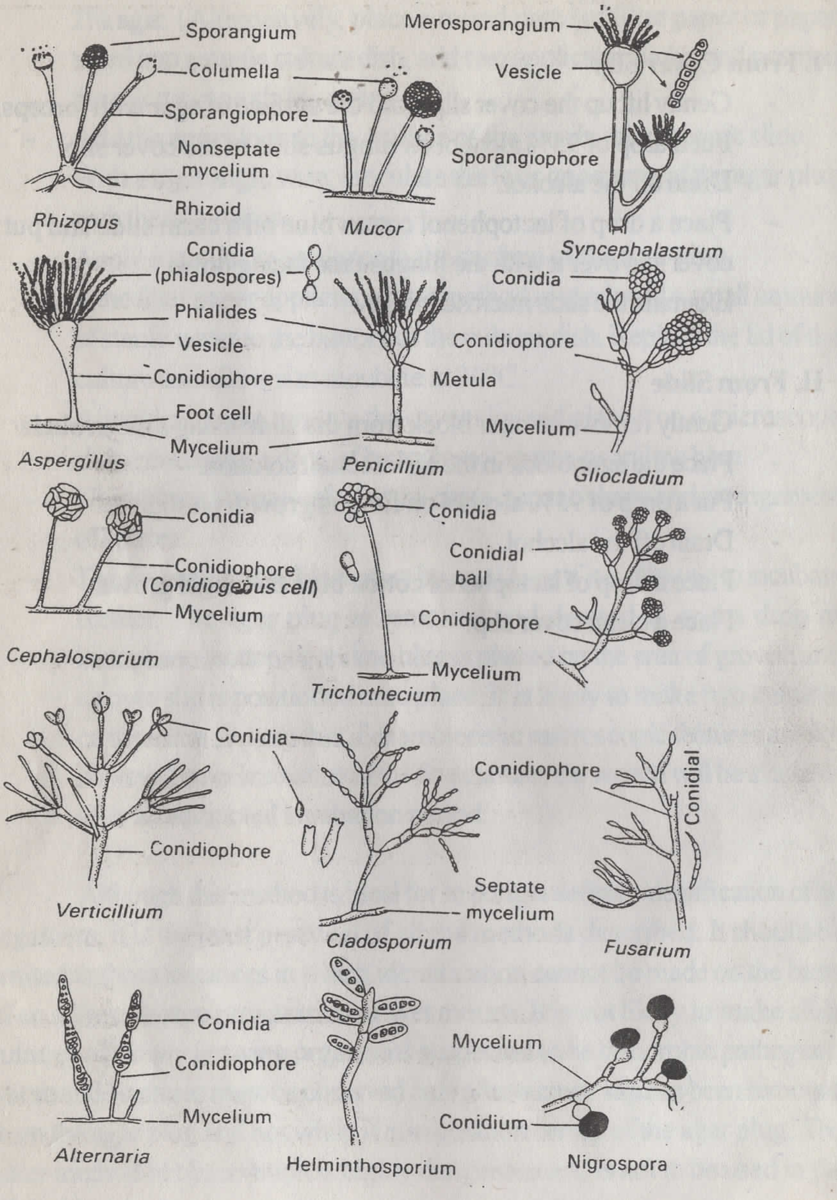

Sporangium
Columella
Sporangiophore
Nonseptate mycelium
Rhizoid
Rhizopus
Merosporangium
Vesicle
Sporangiophore
Mucor
Syncephalastrum
Conidia (phialospores)
Phialides
Vesicle
Conidiophore
Foot cell
Mycelium
Aspergillus
Conidia
Conidiophore
Metula
Penicillium
Mycelium
Gliocladium
Conidia
Conidiophore
(Conidiogenous cell)
Mycelium
Cephalosporium
Conidia
Conidial ball
Conidiophore
Mycelium
Trichothecium
Conidia
Conidiophore
Verticillium
Conidiophore
Septate mycelium
Cladosporium
Conidial
Fusarium
Conidia
Conidiophore
Mycelium
Alternaria
Mycelium
Conidium
Helminthosporium
Nigrospora

Figure 9.1 : Structures of some Fungal Genera that Help in their Identification.

Structures of some Fungal Genera that Help in their Identification.

Zygomycetes:

Rhizopus	-	White to dark gray nonseptate mycelium with root like rhizoids; black columellate sporangiophores, in clusters.
Mucor	-	White to dark gray nonseptate mycelium without rhizoids; single columellate sporangiophores.
Syncephalastrum	-	White to dark gray colonies; aerial sporangiophores with vesicles each bearing several merosporangia; uniseriate 5-10 sporangiospores.

Deuteromycetes:

Aspergillus	-	Greenish-blue, black or green colonies; condiophore arising from a foot-cell, catenate (basipetal) condidia on phialides (1 or 2 series) on vesicle.
Penicillin	-	Greenish or blue green colonies; conidia in long chains on repeatedly branched conidiophores resembling a brushlike head (penicillus).
Gliocladium	-	Dark green condia forming a mucilaginous mass or globlue.
Cephalosporium	-	Cottony gray or rose coloured; condidia head in a mass at the apex of conidiophore (condiogenous cell).
Trichothecium	-	White to pink; two celled condidia loosely held together.
Trichoderma	-	White to green, fast growing; conidia in balls.
Verticillium	-	Pinkish brown, phialides in verticles.
Cladosporium	-	Greenish black and powdery; branched conidiophores; conidia variable.
Fusarium	-	Woolly, white to pink; sickle-shaped transversely septate macroconidia produced in sporodochia.l
Alternaria	-	Grayish green or black; transversely and longitudinally septate (muriform), beaked condidia, in acropetal manner.
Helminthosporium	-	Gray to black; dark large, transversely septate condia.
Nigrospora	-	White to gray; unicellular dark coloured, spherical conidia.

Appendix : I

Table 1 indicates the pH range of each indicator and colour changes which occur in Acidic and Alkaline enyironment.

Indicators	Full Acid Color	Full Alkaline Color	pH Range
Cresol Red	red	yellow	0.2-1.8
Meta Cresol Purple (acid range)	red	yellow	1.2-2.8
Thymol Blue	red	yellow	1.2-2.8
Brom Phenol	yellow	blue	3.0-4.6
BlueBrom Cresol Green	yellow	blue	3.8-5.4
Chlor Cresol Green	yellow	blue	4.0-5.6
Methyl Red	red	yellow	4.4.-6.4
Chlor Phenol Red	yellow	red	4.8-6.4
Brom Cresol Purple	yellow	purple	5.2-6.8
Brom Thymol Blue	yellow	blue	6.0-7.6
Neutral Red	red	amber	6.8-8.0
Phenol Red	yellow	red	6.8-8.4
Cresol Red	yellow	red	7.2-8.8
Meta Cresol Purple (Alk, range)	yellow	purple	7.4-9.0
Thymol Blue (alkaline range)	yellow	blue	8.0-9.6
Cresophthalein	colorless	red	8.2-9.8
Phenolphthalein	colorless	red	8.3-10.0

Table 2 : The indicators can be prepared by (1) dissolving a measured amount of the indicator in 95% ethanol, (2) adding a measured amount of water, and (3) filtering with filter paper. The following chart provides the correct amounts of indicator, alcohol and water for various indicator solutions.

Indicator Solution	Indicator (gm.)	95% Ethanol (ml.)	Distilled H_2O
Brom Cresol Green	0.4	500	500
Brom Cresol Purple	0.4	500	500
Bromo Thymol Blue	0.4	500	500
Cresol Red0.2	0.2	500	500
Methyl Red	0.2	500	500
Phenolphthalain	1.0	50	50
Phenol Red	0.2	500	500
Shymol Blue	0.4	500	500

Appendix : II

Staining Reagents

Acid-Fast Stain

Carbol fuchsin (Ziehl's)

Solution A

Basic fuchsin (90% dye content)	0.3 gm
Ethyl alcohol (95%)	10.0 ml

Solution B

Phenol	5.0 gm
Distilled water	95.0 ml

Note: Mix solutions A and B. Add 2 drops of Triton X per 100 ml of stain for use in heatless method.

Acid Alcohol

Ethyl alcohol (95%)	97.0 ml
Hydrochloric acid	3.0 ml

Methylene Blue (Loeffler's)

Solution A: Dissolve 0.3 gm. Of Methylene blue (90% dye content) in 30.0 ml. ethyl alcohol (95%)

Solution B: Dissolve 0.01 gm. Potassium hydroxide in 100.0 ml. distilled water. Mix solutions A and B.

Capsule Stain

Crystal violet (1%)

Crystal violet (85% dye content)	1.0 gm
Distilled water	100.0 ml

Fungal Stains

Lactophenol-cotton blue solution

Lactic acid	20.0 ml
Phenol	20.0 gm
Glycerol	40.0 ml
Distilled water	20.0 ml
Antiline blue	0.05 gm

Note: Heat gently to dissolve in hot water (double boiler). Then add aniline blue dye.

Water-iodine solution

Gram's iodine (as in Gram's stain)	10.0 ml
Distilled water	30.0 ml

Gram Stain

Crystal violet (Hucker's)

Solution A

Crystal violet (90% dye content)	2.0 gm
Ethyl alcohol (95%)	20.0 ml

Solution B

Ammonium oxalate	0.8 gm
Distilled water	80.0 ml

Note: Mix solutions A and B.

Gram's iodine

Iodine	1.0 gm
Potassium iodide	2.0 gm
Distilled water	300.0 ml

Ethyle alcohol (95%)

Ethyl alcohol (100%)	95.0 ml
Distilled water	5.0 ml

Safranin

Safranin O	0.25 ml
Ethyl alcohol (95%)	10.0 ml
Distilled water	100.0 ml

Negative Stain

Nigrosin

Nigrosin, water-soluble	10.0 gm
Distilled water	100.0 ml

Note : Immerse in boiling water bath for 30 minutes.

Formalin

Note: Filter twice through double filter paper.

Spore Stain

Malachite green

Malachite green	5.0 gm
distilled water	100.0 ml

Appendix : III

Biochemical Test Reagents

Barrit's Reagent, for detection of acetylmethyl-carbinol

Solution A

Alpha-naphthol	5.0 gm
Ethanol, absolute	95.0 ml

Note: Dissolve the alpha-naphthol in the ethanol with constant stirring.

Solution B

Potassium hydroxide	40.0 gm
Creatine	0.3 gm
Distilled water	100.0 ml

Note: Dissolve the potassium hydroxide in 75 ml of distilled water. The solution will become warm. Allow to cool to room temperature. Add the creatine and stir to dissolve. Add the remaining water. Store in a refrigerator.

Biotin-histidine solution, for Ames test

l-Histidine HCl	0.5 mM
Biotin	0.5 mM
Distilled water	10.0 ml

Buffered glycerol (pH 7.2), for immunofluorescence

Glycerin	90.0 ml
Phosphate buffered saline	10.0 ml

Diphenylamine reagent, for detection of nitrates

Dissolve 0.7 gm diphenylamine in a mixture of 60 ml concentrated sulfuric acid and 28.8 ml of distilled water. Cool and slowly add 11.3 ml of concentrated hydrochloric acid. Allow to stand for 12 hours. Sedimentation indicates that the reagent is saturated.

Ferric chloride reagent

Ferric chloride	10.0 gm
Distilled water	100.0 ml

Gram's iodine, for detection of starch

As in Gram's stain

Hydrogen peroxide, 3% for detection of catalase activity

Note: Refrigerate when not in use.

Kavac's reagent, for detection of indole

p-Dimethylaminobenzaldehyde	5.0 gm
Amyl alcohol	75.0 ml
Hydrochloric acid (concentrated)	25.0 ml

Note; Dissolve the p-dimethylaminobenzaldehyde in the amyl alcohol. Add the hydrochloric acid.

McFarland Barium Sulfate Standards, for Staph-Ident procedure

Prepare 1% aqueous barium chloride and 1% aqueous sulfuric acid solutions. Using the foloowing table, add the amounts of barium chloride and sulfuric acid to clean 15 x 150 mm screwcapped test tubes. Label the tubes 1 through 10.

Preparation of McFarland Nephelometer Standards

Tube Number	Barium Chloride	Sulfuric acid1% (ml)	Corresponding Approximate Density of Bacteria (million/ml)
1.	0.1	9.9	300
2.	0.2	9.8	600
3.	0.3	9.7	900
4.	0.4	9.6	1,200

Methyl cellulose, for microscopic observation of protozoa

Methyl cellulose	10.0 gm
Distilled water	90.0 ml

Methyl red solution, for detection of acid

Methyl red	0.1 gm
Ethyl alcohol	300.0 ml
Distilled water	200.0 ml

Note: Dissolve the methyl red in the 95% ethyl alcohol. Dilute to 500 ml with distilled water.

Nessler's reagent, for detection of ammonia

Potassium iodide	50.0 gm
Distilled water (ammonia-free	35.0 ml

Add saturated aqueous solution of mercuric chloride until a slight precipitate persists.

Potassium hydroxide (50% aqueous) 400.0 ml

Note: Dilute to 1000 ml with ammonia-free distilled water. Let stand for 1 week, decant supernatant liquid and store in a tightly capped amber bottle.

Nitrate Test Reagent (Diphenylamine Reagent)

Dissolve 0.7 gm, diphenlamine in a mixture of 60 ml of concentrated sulfuric acid and 28.8 ml. of distilled water.

Cool and add slowly 11.3 ml. of hydrochloric acid (conc.). After the solution has stood for 12 hours some of the base separates, showing that the reagent is saturated.

Nitrite test solution, for detection of nitrites

Solution A, Sulfanilic acid	
Sulfanilic acid	8.0 gm
Acetic acid, 5N: 1 part glacial acetic acid to 2.5 parts distilled water	1000.0 ml
Solution B, Alpha-naphthylamine	
Alpha-napthylamine	5.0 gm
Acetic acid, 5 N	1000.0 ml

Orthonitrophenyl-b-D-galactoside (ONPG), for enzyme induction

0.1 M. sodium phosphate buffer (pH 7.0)	50.0 ml
ONPG (8×10^{-4} M)	12.5 mg

Oxidase test reagent

Mix 1.0 gm. Of dimethyl-p-phenylenediamine hydrochloride in 100 ml. of distilled water.

Preferably, the reagent should be made up fresh, daily. It should not be stored longer than one week in the refrigerator. Tetramethyl-p-phenylenediamine dihydrochloride (1%) is even more sensitive, but is considerably more expensive and more difficult to obtain.

***p*-Aminodimethylanaline oxalate,** for detection of oxidase activity

p-Aminodimethylanaline oxalate	0.5 gm
Distilled water	50.0 ml

Note: to dissolve fully, gently warm the solution.

Phosphate-buffered saline, 1% (pH 7.2-7.4), for immunofluorescence

Solution A	
Disodium phosphate	1.4 gm
Distilled water	100.0 ml
Solution B	
Sodium dihydrogen phosphate	1.4 gm
Distilled water	100.0 ml

Note: Add 84.1 ml of solution A to 15.9 ml of solution B. Add 8.5 gm of sodium chloride and q.s. to 1 liter.

Physiological Saline

Dissolve 8.5 gm, sodium chloride in 1 liter distilled water.

Vaspar

Melt together 1 pound of Vaseline and 1 pound of paraffin. Store in small bottles for student use.

Appendix : IV

Microbiological Media

The formulas of the media are listed alphabetically in grams per liter of distilled water unless otherwise specified. Sterilization of the media is accomplished by autoclaving at 15 lb pressure for 15 minutes unless otherwise specified. Most of the media are available commercially in powdered from, with specific instructions for their preparation and sterilization.

Ammonium sulfate broth (pH 7.3)

Ammonium sulfate	2.0
Magnesium sulfate $7H_2O$	0.5
Ferric sulfate 7H2)	0.03
Sodium chloride	0.3
Magnesium carbonate	10.0
Dipotassium hydrogen phosphate	1.0

Bacteriophage broth 10x (pH 7.6)

Peptone	100.0
Beef extract	30.0
Yeast extract	50.0
Sodium chloride	25.0
Potassium dihydrogen phospharte	80.0

Basal Salts agar* and broth (pH 7.0)

0.5 M Sodium diphosphate	100.0 ml
1.0 M Potassium dihydrogen phosphate	100.0 ml
Distilled water	800.00
0.1 M. Calcium chloride	1.0 ml
1.0 M Magnesium sulfate	10.0 ml

Note: Swirl

Ammonium	2.0
* Agar	15.0

Note: Swirl unit completely dissolved, autoclave, and cool. Aseptically add 10.0 ml of 1% sterile glucose.

Bile esculin (pH.6.6)

Beef extract	3.0
Peptone	5.0
Esculin	1.0
Oxgall	40.0
Ferric citrate	0.5
Agar	15.0

Blood agar (pH 7.4)

Infusion from beef heart	500.00
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0

Note: Dissolve the above ingredients and autoclave. Cool the sterile blood agar base to 45°C to 50°C. Aseptically add 50 ml of sterile defibrinated blood. Mix thoroughly, avoiding accumulation of air bubbles. Dispense into sterile tubes or plates while liquid.

Brain heart infusion (pH 7.4)

Infusion from calf brain	200.0
Infusion from beef heart	250.0
Peptone	10.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
Agar	1.0

Campy BAP agar (pH 7.0)

Trypticase peptone	10.0
Thiotone	10.0
Dextrose	1.0
Yeast extract	2.0
Sodium chloride	5.0
Sodium bisulfide	0.1
Agar	15.0
Vancomycin	10.0 mg
Trimethoprim lactate	5.0 mg

Polymyxin B sulfate	2500.0 IU
Amphotericin B	2.0 mg
Cephalothin	15.0 mg
Defibrinated sheep blood	10.0%

Note: Aseptically add the antibiotics and defibrinated sheep blood to the sterile, cooled, and molten agar.

Chocolate agar (pH 7.0)

Proteose peptone	20.0
Dextrose	0.5
Sodium chloride	5.0
Disodium phosphate	5.0
Agar	15.0

Note: Aseptically and 5.0% defibrinated sheep blood to the sterile and molten agar. heat at 80°C until a chocolate color develops.

Deoxyribonuclease (Dnase) agar (pH 7.3)

Deoxyribonucleic acid	2.0
Phytane	5.0
Sodium chloride	5.0
Trypticase	15.0
Agar	15.0

Endo agar (pH 7.5)

Peptone	10.0
Lactose	10.0
Dipotassium phosphate	3.5
Sodium sulfite	2.5
Basic fuchsin	0.4
Agar	15.0

Eosin-methylene blue agar, Levine (pH 7.2)

Peptone	10.0
Lactose	5.0
Dipotassium phosphate	2.0
Agar	13.5
Eosin Y	0.4
Methylene blue	0.065

Gel diffusion agar

Sodium barbital buffer	100.0 ml
Noble agar	0.8

Clycerole yeast extract agar supplemented with aureomycin (pH 7.0)

Glycerol	5.0 ml
Yeast extract	2.0
Dipotassium phosphate	1.0
Agar	15.0

Note: Aseptically add aureomycin, 10 µg per ml, to the sterile, cooled, and molten agar.

Glucose salts broth (pH 7.2)

Dextrose	5.0
Sodium chloride	5.0
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0

Grape juice broth

Commercial grape or apple juice	
Ammonium biphosphate	0.25%

Note: Sterilization not required when using a large yeast inoculum.

Inorganic synthetic broth (pH 7.2)

Sodium chloride	5.0
Magnesium sulfate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium hydrogen phosphate	1.0

KF broth (pH 7.2)

Polypeptone	10.0
Yeast extract	10.0
Sodium chloride	5.0

Sodium glycerophosphate	10.0
Sodium carbonate	0.636
Maltose	20.0
lactose	1.0
Sodium azide	0.4
Phenol red	0.018

Lactose fermentation broth 1x and 2x* (pH 6.9)

Beef extract	3.0
Peptone	5.0
Lactose	5.0

Note: *For 2x broth use twice the concentration of the ingredients.

Litmus milk (pH 6.8)

Skim milk powder	100.0
Litmus	0.075

Note: Autoclave at 12 lb pressure for 15 minutes.

MacConkey's agar (pH 7.1)

Bacto peptone	17.0
Proteose peptone	3.0
Lactose	10.0
Bile salts mixture	1.5
Sodium chloride	5.0
Agar	13.5
Neutral red	0.03
Crystal violet	0.001

Mannitol Salt agar (pH 7.4)

Beef extract	1.0
Peptone	10.0
Sodium chloride	75.0
d-Mannitol	10.0
Agar	15.0
Phenol red	0.025

M-endo broth (pH 7.5)

Yeast extract	6.0
Thiotone peptone	20.0
Lactose	25.0
Dipotassium phosphate	7.0
Sodium sulfite	2.5
Basic fuchsin	1.5

Note: Heat until boiling, do not autoclave.

M-FC broth (pH 7.4)

Biosate peptone	10.0
Polypeptone peptone	5.0
Yeast extract	3.0
Sodium chloride	5.0
Lactose	12.5
Bile salts	1.5
Aniline blue	0.1

Note: Add 10 ml of rosolic acid (1% in 0.2N sodium hydroxide). Heat to boiling with agitation, do not autoclave.

Milk agar (pH 7.2)

Skim milk powder	100.0
Peptone	5.0
Agar	15.0

Note: Autoclave at 12 lb pressure for 15 minutes.

Minimal agar (pH 7.0)

Aminmal agar, supplemented with streptomycin and thiamine*

Solution A (pH 7.0)	
Potassium dihydrogen phosphate	3.0
Disodium hydrogen phosphate	6.0
Ammonium chloride	2.0
Sodium chloride	5.0
Distilled water	800.0 ml
Solution B (pH 7.0)	
Glucose	8.0
Magnesium sulfate $7H_2O$	0.1
Agar	15.0

Distilled water	200.0ml

Note: Autoclave solutions A and B separately and combine.

*To solution B add 0.001 gm of thiamine prior to autoclaving. To the combined sterile and molten medium, add 50 mg (1 ml of 50 mg per ml) sterile streptomycin solution before pouring agar plates.

MR-VP broth (pH 6.9)

Peptone	7.0
Dextrose	5.0
Potassium phosphate	5.0

Muleller-Hinton agar (pH 7.4)

Beef, infusion	300.0
Casamino acids	17.5
Starch	1.5
Agar	17.0

Mueller-Hinton tellurite agar (pH 7.4)

Casamino acids	20.0
Casein	5.0
1-Tryptophane	0.05
Potassium dihydrogen phosphate	0.3
Magnesium sulfate	0.1
Agar	20.0

Note: Aseptically add 12.5 ml of tellurite serum to the sterile, 50°C Molten agar.

Nitrate broth (pH 7.2)

Sodium nitrite	2.0
Magnesium sulfate 7H2O	0.5
Feeric Sulfate 7H2O	0.03
Sodium chloride	0.3
Sodium carbonate	1.0
Dipotassium hydrogen sulfate	1.0

Nitrite Meidum (for Nitrobacter)

$NaNO_2$	1.0 gm.

$MgSO_4, 7H_2O$	0.5 gm.
$FeSO_4, 7H_2O$	0.03 gm
NaCl	0.3 gm.
Na_2CO_3	1.0 gm
K_2HPO_4	1.0 gm
Water	1000.0 ml.

For the isolation of Nitrobacter from soil, sterilization is not necessary if the inoculations are made as soon as the medium is made up. The pH should be adjusted to 7.3. If sterilization is desirable for storage for other reasons, adjust the pH aseptically after sterilization with sterile IN HCl.

Nitrogen-Free Medium (for Azotobacter)

K_2HPO_4	0.5 gm.
$MgSo_4, 7H_2O$	0.2 gm.
NaCl	0.2 gm.
$FeSO_4, 7H_2O$	0.001 gm.
$MnSO_4, 4H_2O$	0.005 gm.
$CaCo_3$	10.0 gm.
Molybdenum salt	trace
*Mannitol	10.0 gm.
Water	1000.0 ml.

*Sterilize separately.

Adjust pH to 7.2

If this medium is to be used immediately to isolate Azotobacter from soil, sterilization is not necessary. When it must be stored for any length of time it should be sterilized. If it is to be sterilized, the mannitol should be dissolved separately in 100 ml. of water and sterilized at 121°C for 20 minutes. The remainder of the medium is sterilized in a similar manner. After sterilization the two solutions are mixed aseptically the dispensed in 8 ox. Prescription bottles (50 ml. per bottle).

Nitrogen-free mannitol agar* and broth (pH 7.3)

Mannitol	15.0
Dipotassium hydrogen phosphate	0.5

Magnesium sulfate	0.2
Calcium sulfate	0.1
Sodium chloride	0.2
Calcium carbonate	5.0
*Agar	15.0

Nutrient agar* and broth (pH 7.0)

Peptone	5.0
Beef extract	3.0
*Agar	15.0

Nutrient gelatin (pH 6.8)

Peptone	5.0
Beef extract	3.0
Gelatin	120.0

Peptone broth (pH 7.2)

Peptone	4.0

Phenol red dextrose broth (pH 7.3)

Trypticase	10.0
Dextrose	5.0
Sodium chloride	5.0
Phenol red	0.018

Note: Autoclave at 12 lb pressure for 15 minutes.

Phenol red inulin broth (pH 7.3)

Trypticase	10.0
Inulin	5.0
Sodium chloride	5.0
Phenol red	0.018

Note: Autoclave at 12 lb pressure for 15 minutes.

Phpenol red inulin broth (pH 7.3)

Trypticase	10.0
Inulin	5.0
Sodium chloride	5.0

Phenol red	0.018

Note: Autoclave at 12 lb pressure for 15 minutes.

Phpenol red lactose broth (pH 7.3)

Trypticase	10.0
Lactose	5.0
Sodium chloride	5.0
Phpenol red	0.018

Note: Autoclave at 12 lb pressure for 15 minutes.

Phenol red sucrose broth (pH 7.3)

Trypticase	10.0
Sucrose	5.0
Sodium chloride	5.0
Phpenol red	0.018

Note: Autoclave at 12 lb pressure for 15 minutes.

Phenylethyl alcohol agar (pH 7.3)

Trypticase	15.0
Phytane	5.0
Sodium chloride	5.0
β-phenylethyl alcohol	2.0
Agar	15.0

Sobouraud agar (pH 5.6)
Sabouraud agar supplemented with aureomycin*

Peptone	10.0
Dextrose	40.0
Agar	15.0

Note: *Aseptically add aureomycin, 10 μg per ml, to the sterile, cooled, and molten medium.

Salt medium – *Halobacterium*

Sodium chloride	250.0
Magnesium sulfare 7H2O	10.0
Potassium chloride	5.0

Calcium chloride 5H2O	0.2
Yeast extract	10.0
Tryptone	2.5
Agar	20.0

Note: The quantities given are for preparation of one liter final volume of the medium. In preparation, make up two solutions, one involving the yeast extract and tryptone, and the other the salts. Adjust the pH of the nutrient solution to 7. Sterilize separately. Mix and dispense aseptically.

SIM agar (pH 7.3)

Peptone	30.0
Beef extract	3.0
Ferrous ammonium sulfate	0.2
Sodium thisulfate	0.025
Agar	3.0

Simmons citrate agar (pH 6.9)

Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulfate	0.2
Agar	15.0
Brom thymol blue	0.08

Snyder teset agar (pH 4.8)

Tryptone	20.0
Dextrose	20.0
Sodium chloride	5.0
Brom cresol green	0.02
Agar	20.0

Sodium Chloride broth, 5.6% (pH 7.0)

Brain heart infusion broth	100.0 ml
Sodium chloride	6.5

Starch agar (pH 7.0)

Peptone	5.0
Beef extract	3.0
Starch (soluble)	2.0
Agar	15.0

Thioglycollate, fluid (pH 7.1)

Peptone	15.0
Yeast extract	5.0
Dextrose	5.0
1-Cystine	0.75
Thioglycollic acid	0.3 ml
Agar	0.75
Sodium chloride	2.5
Resazurin	0.001

Tinsdale agar (pH 7.4)

Proteose peptone, No.3	20.0
Sodium chloride	5.0
Agar	20.0

Note: Following boiling, distribute in 100 ml flasks. Autoclave, cool to 55°C and 15 ml of rehydrated Tinsdale enrichment to each 100 ml, and mix thoroughly before dispensing.

Top agar (for Ames test)

Sodium chloride	5.0
Agar	6.0

Tributyrin agar (pH 7.2)

Peptone	5.0
Beef extract	3.0
Agar	15.0
Ttriburyrin	10.0

Note: Dissolve peptone, beef extract, and agar while heating. Cool to 90°C, add the tributyrin, and emulsify in a Wring blender.

Triple sugar-iron agar (pH (7.4)

Beef extract	3.0

Yeast extract	3.0
Peptone	15.0
Proteose peptone	5.0
Lactose	10.0
Saccharose	10.0
Dextrose	1.0
Ferrous sulfate	0.2
Sodium chloride	5.0
Sodium thiosulfate	0.3
Phenol red	0.024
Agar	12.0

Trypticase nitrate broth (pH 7.2)

Trypticase	20.0
Disodium phosphate	2.0
Dextrose	1.0
Agar	1.0
Potassium nitrate	1.0

Trypticase soy agar (pH 7.3)

Trypticase	15.0
Phytane	5.0
Sodium chloride	5.0
Agar	15.0

Tryuptone agar* and broth

Tryptone	10.0
Calcium chloride (reagent)	0.01-0.03 M
Sodium chloride	5.0
*Agar	11.0

Tryptone soft agar

Tryptone	10.0
Potassium chloride (reagent)	5.0 ml
Agar	9.0

Urea broth

Urea broth concentrate (filter-sterilized solution)	10.0 ml
Sterile distilled water	90.o0 ml

Note: Aseptically add the urea broth concentrate to the sterilized and colled distilled water. Under aseptic conditions, dispense 3-ml amounts into sterile tubes.

Yeast extract broth (pH 7.0)

Peptone	5.0
Beef extract	3.0
Sodium chloride	5.0
Yeast extract	5.0

Appendix - V

Some of the Anti-microbial agents, their mode of action and their practical uses.

Agent	Mode of Action	Uses
Phenolic Compounds Phenol	1. Germicidal effect caused by alterationof protein structure resulting in protein denaturation. 2. Surface-active agent (surfactant) precipitates cellular proteins and disrupts cell membranes. (Phenol has been replaced by better disinfectants tht are less irritating, less toxic to tissues, and better inhibitors of micro-organisms).	1. 5% solution: Disinfection. 2. 0.5% to 1% solution: Antiseptic effect and relief of itching as it exerts a local anesthetic effect on sensory nerve endings.
Cresols	1. Similar to phenol. 2. Poisonous and must be used externally. 3. 50% solution of cresols in vegetable oil, known as Lysol.	2% to 5% Lysol solutions used as disinfectants.
Haexachlorophene	Germicidal activity similar to phenol. (This agent is to be used with care, especially on infants, because after absorption it may cause neuro-toxic effects)	1. Reduction of pathogenic organisms on skin; added to detergents, soaps, lotions, and creams. 2. Effective against gram-positive organisms. 3. An antiseptic used topically.
Resorcinol	1. Germicidal activity similar to that of phenol. 2. Acts by precipitating cell protein.	1. Antiseptic. 2. Keratolytic agent for softening or dissolving keratin in epidermis.

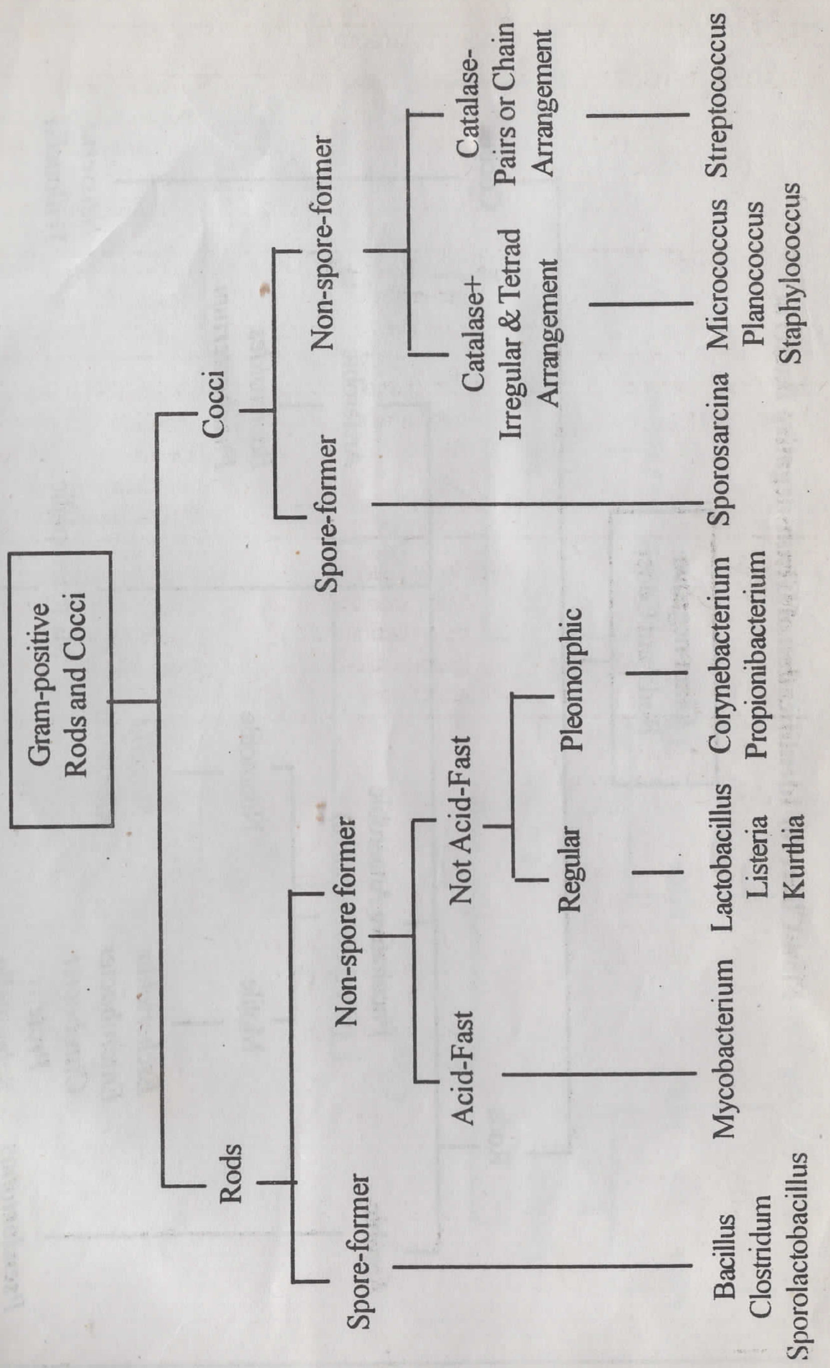
Agent	Mode of Action	Uses
Hexylresorcinol	Germicidal activity similar to that of phenol.	1. Treatment of worm infections of phenol. 2. Urinary antiseptic.
Thymol	1. Related to the cresols. 2. More effective than phenol.	1. Antifungal activity. 2. Treatment of hookworm infections. 3. Mouthwashes and gargle solutions.
Alcohols Ethyl: C_2H_5OH Isopropyl: $(CH_3)_2$ CHOH	1. Lipid solvent. 2. Denaturation and coagulationof proteins. 3. Wetting agent used in tinctures to increase the wetting ability of other chemicals. 4. Germicidal activity increases with increasing molecular weight.	Skin antiseptics; Ethyl-50% to 70% Isopropyl-75%
Halogens Chlorine compounds: Sodium hypochlorite (Dakin's fluid): NaOcl Chloramine: $CH_3C_6H_4SO_2NNaCl$	1. Germicidal effect resulting from rapid combination with proteins. 2. Chlorine reacts with water to form hypochlorous acid, which is bactericidal. 3. Oxidizing agent. 4. Noncompetitively inhibits enzymes, especially those dealing with glucose metabolism, by reacting with SH and NH_2 groups on the enzyme molecule.	1. Water purification. 2. Sanitation of utensils in dairy and restaurant industries. 3. Chloramine, 0.1% to 2% solutions, for wound irrigation and dressings. 4. Microbicidal.

Agent	Mode of Action	Uses
Iodine compounds: Tincture of iodine Povidone-iodine solution (Betadine)	1. Mechanism of action is not entirely known, but it is believed that it precipitates proteins. 2. Surface-active agent.	1. Tinctures of iodine are used for skin antisepsis. 2. Treatment of goiter. 3. Effective against spores, fungi, and viruses.
Heavy Metals Mercury compounds: Inorganic: Mercury bichloride Mercurial ointments	1. Mercuric ion brings about precipitation of cellular proteins. 2. Noncompetitive inhibition of specific enzymes caused by reaction with sulfhydryl group (SH) on enzymes of bacterial cells.	1. Inorganic mercurials are irritating to tissues, toxic systemically, adversely affected by organic matter, and they have no action on spores. 2. Mercury compounds are mainly used as disinfectants of laboratory materials.
Organic mercurials: Mercurochrome (merbromin) Merthiolate (thimerosal) Metaphen (nitromersol) Merbak (acetomeroctol)	1. Similar to those of inorganic mercurials, but in proper concentrations are useful antiseptics. 2. Much less irritating than inorganic mercurials.	1. Less toxic, less irritating; used mainly for skin asepsis. 2. Do not kill spores.
Silver compounds: Silver nitrate	1. Precipitate cellular proteins. 2. Interfere with metabolic activities of microbial cells. 3. Inorganic salts are germicidal.	Asepsis of mucous membrane of throat and eyes.
Surface-Active Agents Wetting agents: Emulsifiers, soaps, and detergents	1. Lower surface tension and aid in mechanical removal of bacteria and soil. 2. If active portion of the agent carries a negative electric charge, it is called an anionic surface-active agent. If active portion of the agent carries a positive electric charge, it is called a cationic surface-active agent. 3. Exert bectericidal activity by interfering with or by depressing metabolic activities of microorganisms. 4. Disrupt cell membranes. 5. Alter cell permeability.	Weak action against fungi, acidfast microorganisms, spores, and viruses.
Cationic agents: Quaternary ammonium compounds Benzalkonium chloride	1. Lower surface tension because of keratolytic, detergent, and emulsifying properties. 2. Their germicidal activities are reduced by soaps.	1. Bactericidal, fungicidal; inactive against spores and viruses. 2. Asepsis of inteact skin. 3. Disinfectant for operating-room equipment. 4. Dairy and restaurant sanitization.
Anionic agents: Tincture of green soap Sodium tetradecyl sulfate	1. Neutral or alkaline salts of high-mulecular-weight acids. Common soaps included in this group. 2. Exert their maximum activity in an acid medium and are most effective against gram-positive cells. 3. Same as all surface-active agents.	1. Cleansing agent. 2. Sclerosing agent in treatment of varicose veins and internal hemorrhoids.
Acids (H^+) Alkali (OH^-)	1. Destruction of cell wall and cell membrane. 2. Coagulation of proteins.	Disinfection, however, of little practical value.

Agent	Mode of Action	Uses
Formaldehyde (liquid or gas)	Alkylating agent causes reduction of enzymes.	1. Room disinfection. 2. Alcoholic solution for instrument disinfection. 3. Specimen preservation.
Ethylene Oxide	Alkylating agent causes reduction of enzymes.	Sterilization of heat-labile materials.
Beta-Propiolactone (liquid or gas)	Alkylating agent causes reduction of enzymes.	1. Sterilization of tissue for grafting. 2. Destruction of hepatitis virus. 3. Room disinfection.
Basic Dyes Crystal violet	Affinity for nucleic acids; interfere with reproduction in gram-positive organisms.	1. Skin antiseptic. 2. Laboratory isolation of gram negative bacteria.

Appendix - VI

Flow Chart for Identification of Gram-positive Bacteria


Gram-positive Rods and Cocci
Rods
Cocci
Spore-former
Non-spore former
Acid-Fast
Not Acid-Fast
Regular
Pleomorphic
Spore-former
Non-spore-former
Catalase+ Irregular & Tetrad Arrangement
Catalase- Pairs or Chain Arrangement
Bacillus
Clostridium
Sporolactobacillus
Mycobacterium
Lactobacillus
Listeria
Kurthia
Corynebacterium
Propionibacterium
Sporosarcina
Micrococcus
Planococcus
Staphylococcus
Streptococcus

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